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14. ABSTRACT
The hypothesis of this proposal is that Latent TGF-beta Binding Protein 1 (LTBP1) is a pro-metastatic factor in breast cancer. To test this hypothesis we proposed three aims 1) Determine the utility of LTBP1 expression as a biomarker in human breast cancer 2) Determine the consequences of modulating LTBP1 levels on cell motility, invasion and metastasis in breast cancer cell lines 3) Investigate the pathobiological effects of gain and loss of LTBP1 in genetically engineered mouse models. Our most important findings are that
1) elevated LTBP1 expression is associated with poor outcome in Basal and HER2 positive ER-negative breast cancers whereas other LTBP genes are more highly expressed in luminal cancers and associated with better outcome; 2) LTBP1S is elevated in highly metastatic mesenchymal-like triple negative breast cancer cells which specific TGF-beta ligands and receptors and the LTBP binding partners Fibronectin and Fibrillin; and 3) Knock down of LTBP1 in breast cancer cell line reduces cell invasion in transwell assays. 4) Metastatic mouse tumors C3(1)-Tag and MMTV-PYMT express elevated LTBP1.

15. SUBJECT TERMS
Cell Adhesion, Involution, Metastasis, Latent TGFbeta Binding Protein, Pregnancy-associated
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1. INTRODUCTION

A large body of literature supports the concept that TGF-beta functions as a tumor suppressor in early breast cancers but at later stages contributes to tumor spread by promoting epithelial to mesenchymal transition. TGF-beta ligands are secreted in a latent form and tethered to the extracellular matrix by Latent TGF-beta binding protein 1 (LTBP1). LTBP1 therefore determines the site of subsequent cytokine signaling and is also required for integrin-mediated stretch activation of this cytokine. Despite these critical roles in TGF-beta biology LTBP1 has not been studied in the mammary gland. This proposal set out to test if LTBP1 plays a role in promoting metastasis by examining LTBP1 expression and testing whether altering LTBP1 levels affect invasion and metastasis in breast cancer.

2. KEYWORDS

Cell-matrix Adhesion, Involution, Metastasis, Latent TGF-beta Binding Protein, Pregnancy-associated Breast Cancer

3. ACCOMPLISHMENTS

What were the major goals of the project:

Task 1) Determine the utility of LTBP1 expression as a biomarker in human breast cancer. (complete)
Task 2) Determine the consequences of modulating LTBP1 levels on cell motility, invasion and metastasis in human and murine breast cell lines. (complete)
Task 3) Determine the pathological significance of gain and loss of LTBP1 in genetically engineered mouse models. (75% complete)

What was accomplished under these goals:

Task 1. Determine the expression of LTBP1 in human and mouse breast cancers and breast cancer cell lines.

Expression in normal mammary gland. LTBP1 integrates into the extracellular matrix surrounding ducts and was intuitively thought to be produced by basal or stromal cells. Contrary to this prediction we found that LTBP1 is produced by luminal cells lining the inside of the mammary ductal system (data is appended in the attached published manuscript Chandramouli et al.). We determined the *Ltbp1* promoter activity pattern over the course of embryonic (Chandramouli et al. Figures 2, 3, 4 a, b and 6), nipple (Figures 4 c, d and 5) and adult (Chandramouli et al. Figures 7 c and d, 8, 9, 10) mammary development using an *Ltbp1-lacZ* reporter mouse. By qPCR we found expression of both long and short isoforms of Ltbp1 mRNA (Chandramouli et al. Figures. 7a, b). We localized the secreted LTBP1 protein by immunofluorescence to the elastic encasement of the mammary ducts (Chandramouli et al. Figures10 g and h, 11). The key findings of this study are that Ltbp1 is produced by a subset of quiescent luminal cells (Chandramouli et al. Figure 7 c-f) exclusively localized to the permanent portion of the ductal system and is absent from proliferating terminal end buds and differentiated

alveoli. It is therefore a rare and valuable marker that is uniquely capable of distinguishing ductal from alveolar luminal lineages. In this location Ltbp1 presents TGF-beta to cells within the basal layer of the permanent ductal system that includes stem cells. By association with elastic fibers it can serve as a mechanosensor. In contrast, temporary side branches are surrounded by Ltbp1 but not elastin and differentiated alveoli lack both of these proteins. These data show that different segments of the mammary tree are surrounded by distinct extracellular matrices that likely influence their cell fate, potency and differentiation status. Ltbp1 expression is dynamically regulated over the course of mammary development. It correlates with lumen formation and the emergence of the luminal cell fate and is maximal during involution after weaning. This developmental period, which involves removal of redundant alveoli and side-branches and extensive matrix remodeling is associated with heightened breast cancer risk associated with the generation of a prometastatic microenvironment. The production of the Ltbp1-TGF-beta complex by luminal cells has consequences for our understanding of the mechanism of breast cancer spread as it suggests that luminal cells, which are the source of all subtypes of human breast cancer, secrete their own prometastatic soil.

In addition to *Ltbp1*, there are three other *Ltbp* genes. To provide a comprehensive understanding of their potentially interrelated roles and establish whether their products act redundantly we investigated the expression of *Ltbp* 2-4 by qRT-PCR analysis over the course of normal mammary development (Figure 1). *Ltbp3* is expressed in a similar developmental pattern to that of *Ltbp1* being markedly elevated during involution. In contrast, *Ltbp3* and *Ltbp4* are expressed at extremely low levels and remain constant.

To ascertain which mammary cell types express these *Ltbp* genes we isolated cells from normal mammary tissue and sorted them into the three major populations by fluorescent activated cell sorting (FACS) based on their CD49f and CD24 surface marker expression. Enrichment for basal, luminal and stromal subpopulations was confirmed by PCR analysis of mRNA for their specific intermediate filaments Keratin (K) 14, K8 and Vimentin respectively. PCR analysis of Ltbp mRNA expression established that Ltbp1L and 1S are enriched in luminal cells, Ltbp2 is expressed in basal and stromal cells, Ltbp3 is expressed in basal and luminal and Ltbp4 in stromal cells (Figure 2).

LTBP expression in human breast tumors and relationship to patient outcome. To test the central hypothesis that increased LTBP1 is prometastatic we performed in silico analyses of *LTBP1* expression in human breast cancers and cell lines and correlated this with patient outcome using the publicly available online database Kaplan-Meier plotter (kmplot.com). *LTBP1* expression in total breast cancer correlated with poor survival in terms of Recurrence-Free Survival (RFS) and Distant Metastasis-Free Survival (DMFS) (Figure 3).
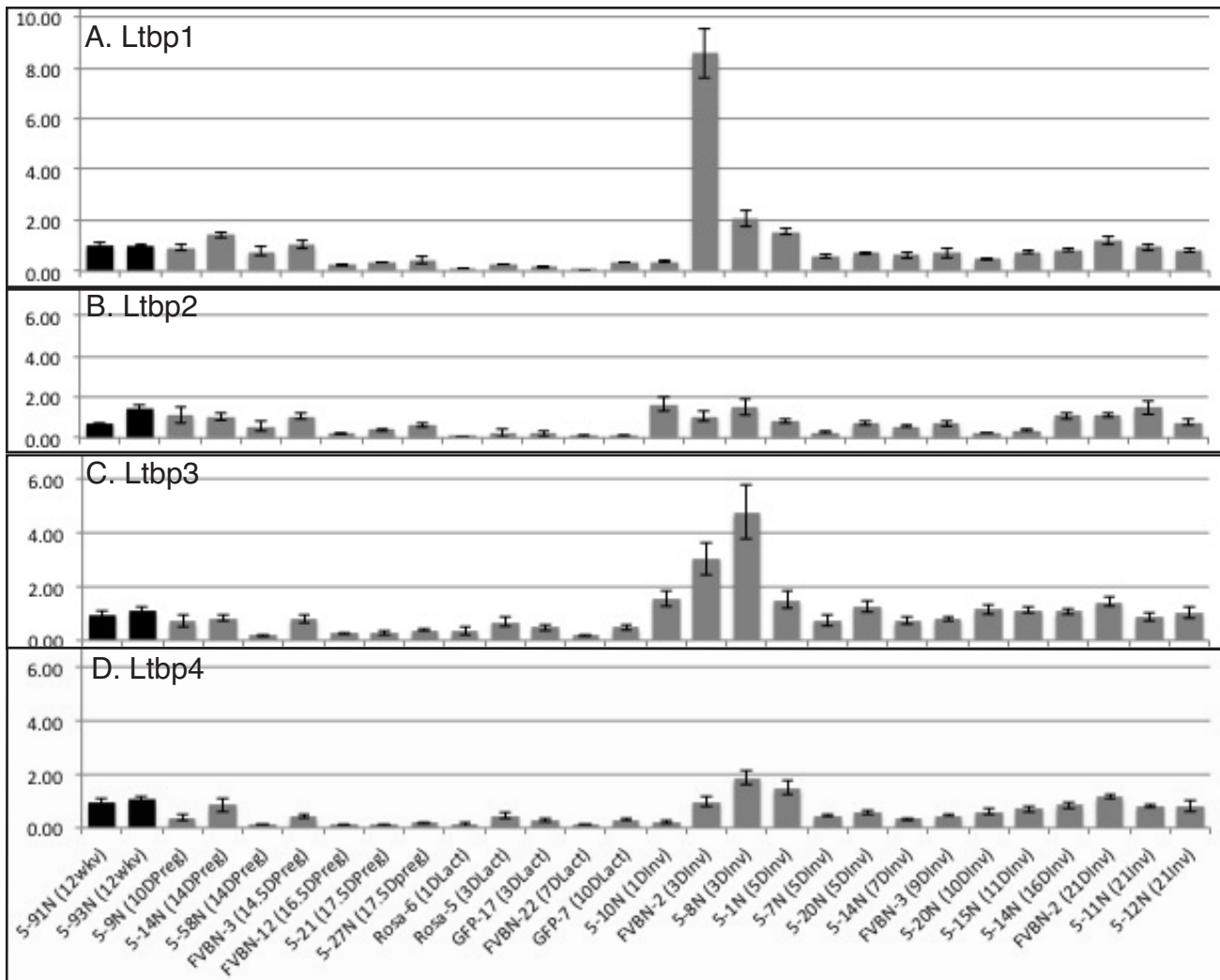
Figure 1: Latent TGFbeta Binding Proteins Ltbp 1-4 Expression in the Postnatal Mammary Gland.
(A) Total RNA from mammary gland harvested from various developmental time points (12-week virgin (wkV), 10, 14.5, and 17.5 days of pregnancy (DPreg), after 3, 7 and 10 days of lactation (DLact), 1, 3, 5,7,9,10, 16 and 21days of involution (Dinv) was reverse transcribed and subjected to qRT-PCR. Ltbp mRNAs were normalized to B2-microglobulin expression and plotted as levels relative to tissue from 12-week-old virgins. Error bars indicate standard deviation of the cycle threshold (Ct) values (n 4). Numbers below indicate the specific mice. Ltbp1 mRNA expression is highly elevated during early involution peaking 3 days after forced pup weaning. (B) Ltbp2 shows a low relatively constant level of expression.(C)Ltbp3 is elevated during early involution peaking 3 days after forced pup weaning (D) Ltbp4 is relatively constant throughout mammary development.
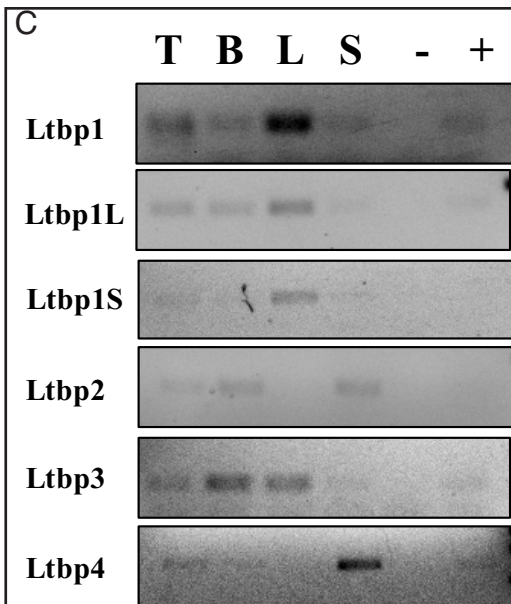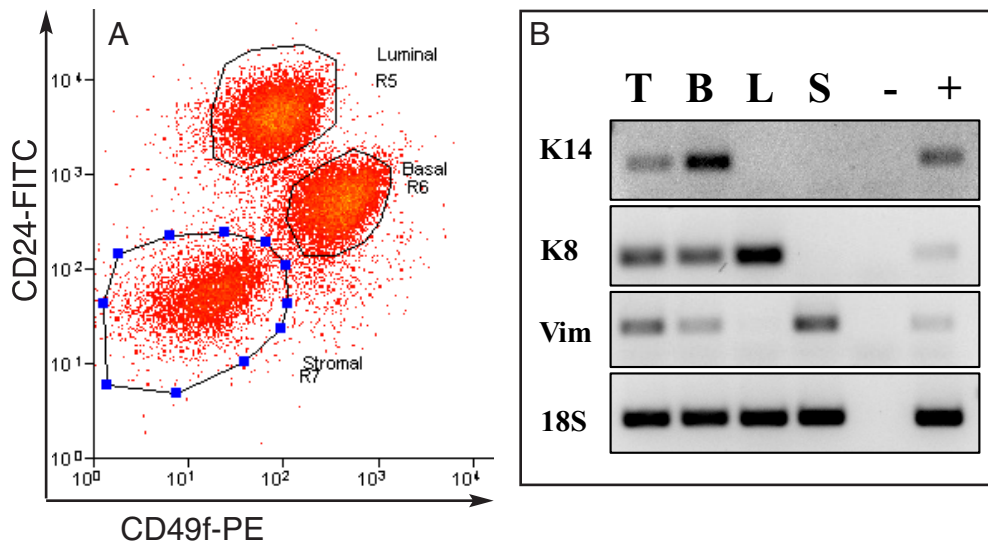
Figure 2: Ltbp Expression in Mammary Cell Subpopulations.

(A) Basal (B), Luminal (L) and Stromal (S) mammary subpopulations: gated on the basis of CD24 and CD49f marker expression by FACS.

(B) PCR characterization of mRNA expression of Keratin 14 (K14), (K8) and vimentin (Vim) markers within total (T), basal (B), (L) luminal and (S) stromal cell subpopulations as gated in (A). 18S rRNA served as a loading control. Control (-) indicates the negative control with no cDNA template added and (+) indicates the positive control cDNA derived from a MMTV-Wnt tumor.

(C) PCR characterization of mRNA expression for Ltbp1-4 as indicated within the same subpopulations characterized above. Note Ltbp1, 1L and 1S are enriched in luminal cells. Ltbp2 is expressed in basal and stromal cells, Ltbp3 is expressed in basal and luminal and Ltbp4 in stromal cells
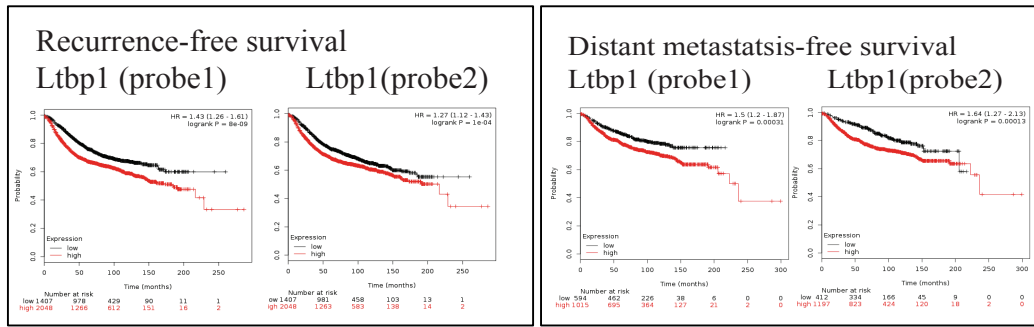
Figure 3: RFS and DMFS plotted for patient samples showing high LTBP1 expression (red) and lower expression (black) as assessed using Kaplan-Meier plotter. These data indicate a correlation between elevated LTBP1 expression and poor outcome.

Analysis of the outcome data broken down into the major subtypes of breast cancer showed that high *LTBP1* expression correlated significantly with recurrence in Basal and HER2 subtypes and showed only a modest difference in luminal A and B subtypes (Figure 4).
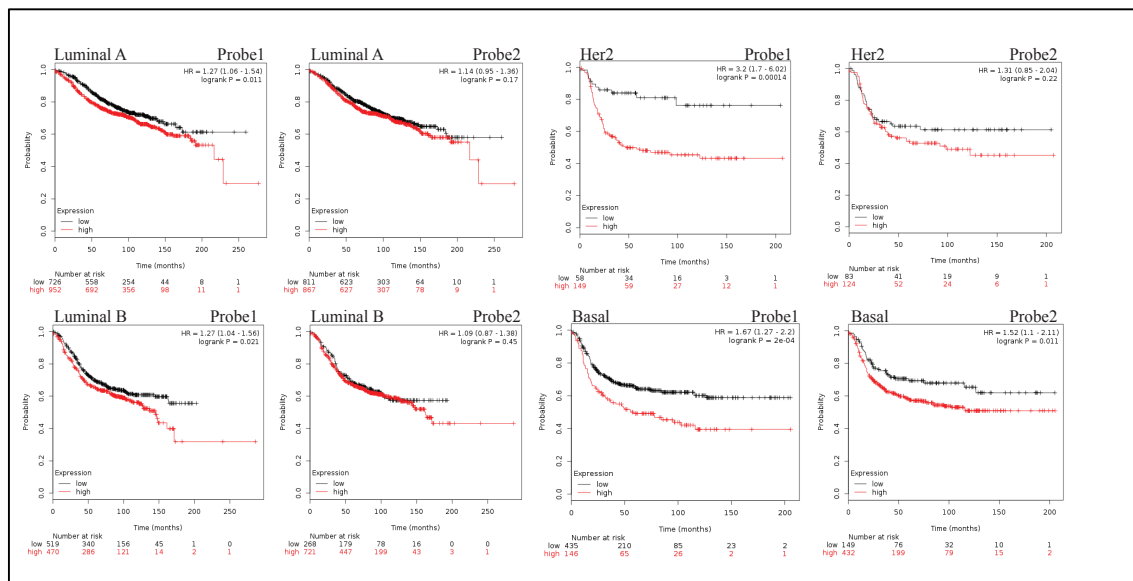

Figure 4: RFS plotted for patient samples showing high LTBP1 expression (red) and lower expression (black) as assessed using Kaplan-Meier plotter. These data indicate a correlation between elevated LTBP1 expression and poor outcome within the HER2 and Basal type breast cancers.

Investigation of the BreastMark database (http://glados.ucd.ie/BreastMark/index.html) showed a subtle association between high *LTBP1* expression and poor outcome in terms of disease-free survival (DFS) in breast cancer as a whole and confirmed a strong significant association with poor outcome within Basal and HER2 subtypes (Figure 5).
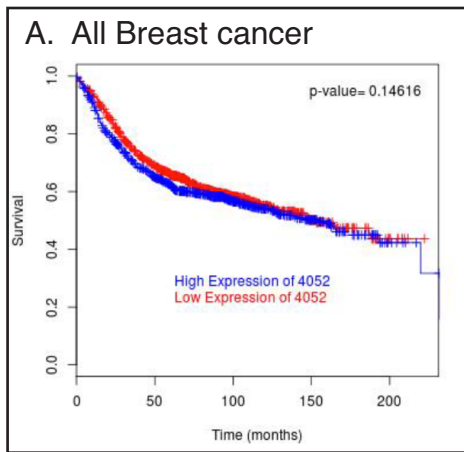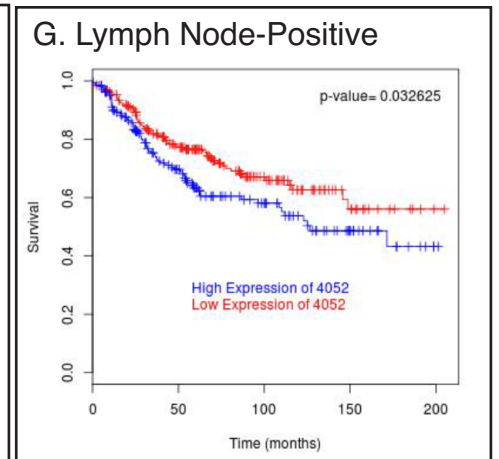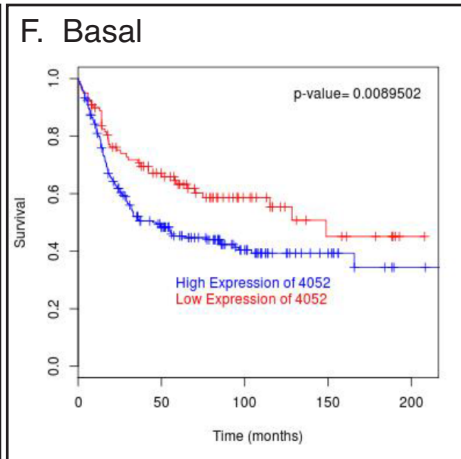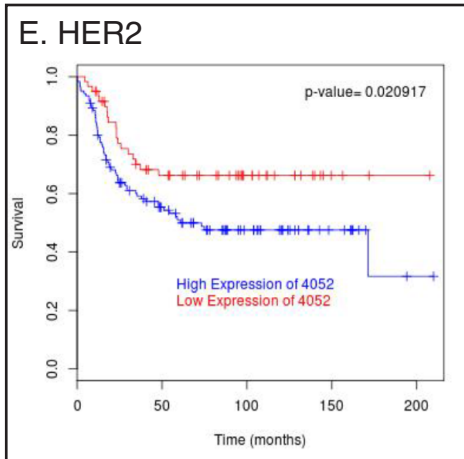
A. All Breast cancer

p-value= 0.14616

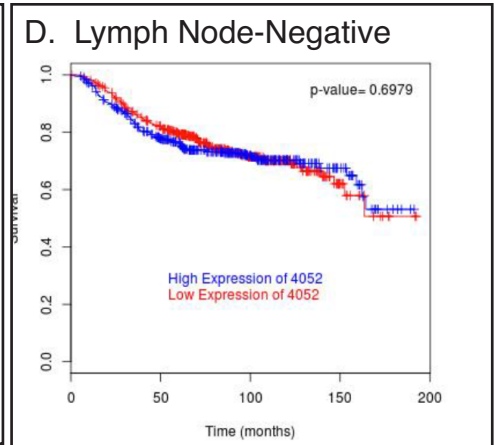High Expression of 4052
Low Expression of 4052

Figure 5: Analysis of LTBP1 expression and Disease-Free Survival DFS in BreastMark Database

High LTBP1 expression is associated with worse outcome (DFS) in Basal, HER2, and Lymph Node (LN)-positive breast cancers. Association between LTBP1 expression and DFS was assessed using the online BreastMark tool that divides patients at the median for high (blue) and low (red) LTBP1 expression. N=number of samples, Hazard Ratio and P value are as indicated. No significant association was found in Luminal A, Luminal B or LN-negative breast tumors (B-D). However, high LTBP1 expression identifies a subset of patients with significantly worse outcome in Basal and HER2 subtypes and those with LN-positive tumors.

B. Luminal A

p-value= 0.13359

High Expression of 4052
Low Expression of 4052

C. Luminal B

p-value= 0.46896

High Expression of 4052
Low Expression of 4052

D. Lymph Node-Negative

p-value= 0.6979

High Expression of 4052
Low Expression of 4052

E. HER2

p-value= 0.020917

High Expression of 4052
Low Expression of 4052

F. Basal

p-value= 0.0089502

High Expression of 4052
Low Expression of 4052

G. Lymph Node-Positive

p-value= 0.032625

High Expression of 4052
Low Expression of 4052

To further examine *LTBP1* expression in different breast cancer subtypes we used publicly available gene expression datasets in the Cancer Genomic Browser (https://genome-cancer.ucsc.edu) (Figure 6). *LTBP1* expression is consistently higher in Basal and HER2 cancers than Luminal A and B (ANOVA p<0.0001). LTBP1 expression is higher in Estrogen Receptor (ER) negative tumors (p<0.0001) and this finding is repeated in nine other data sets. LTBP1 is also significantly higher in triple-negative breast cancers (Figure 6).
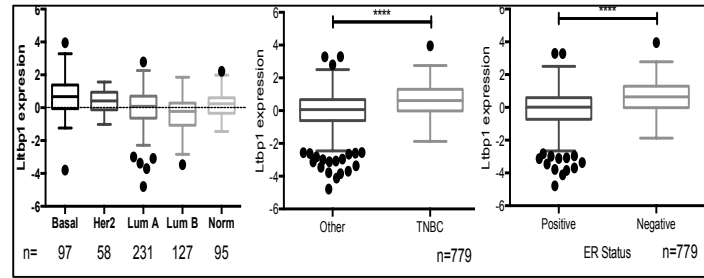

Figure 6: Expression of *LTBP1* correlated with subtype and hormone receptor status.
Left: *LTBP1* expression normalized across breast cancer subtypes (ANOVA p<0.0001). Middle: LTBP1 expression in triple-negative tumors versus all other breast cancers. Right: LTBP1 expression in estrogen receptor positive vs negative tumors. **** = p<0.0001

We interrogated databases of TNBC human breast tumor that distinguish subtypes (TNBCtype). Our results show that total LTBP1 is more highly expressed in TNBC tumors compared to all others and there is a trend towards higher expression on average within the mesenchymal stem-like subgroup, which is the most highly metastatic form with the worse outcome (Figure 7).

Collectively this data supports the concept that high LTBP1 expression is a biomarker for poor prognosis in breast cancer, particularly within Basal and Her2 subtypes. These represent the breast cancers with the worst outcome and in the case of Basal breast cancer are the ones that are prevalent in young women of reproductive age.

In contrast, *LTBPs 2-4* show the opposite of *LTBP1* in terms of their expression levels in different breast cancer subtypes. *LTBP 2-4* are more highly expressed in ER-positive tumors and in Luminal A subtypes (Figure. 8). Moreover, in marked contrast, to the association between high *LTBP1* expression and poor outcome, we found the opposite was true for other members of the LTBP family. High *LTBP 2, 3 or 4* expression was associated with improved outcome in terms of RFS for breast cancer overall in both Kaplan Meier plotter and breastmark databases (Figure 9). This association with better outcome is particularly significant within luminal subtypes in terms of RFS (Figure 10).

A complex scenario was found in terms of DMFS (Figure. 11): High *LTBP3* expression was associated with slightly better outcome in luminal and basal subtypes. High *LTBP2* and *LTBP4* expression was associated with worse outcome. High expression of any *LTBP* was associated with worse outcome in HER2 breast cancer (Figure 11).

Figure 7: LTBP1 Expression is Higher in Triple Negative Breast Cancers.
(A) LTBP1 expression in TNBC versus all other breast cancers in the TGAC database (A) and (B)Hess   et al. and (C) TNBCtype database (http: tnbc.mc.vanderbilt.edu. index.php) BL1:Basal-like1, BL2:Basallike2, IM:Immunomodulatory, M:Mesechymal, MSL:Mesenchymal- stem-like,LAR:Luminal   Androgen Receptor, NS:nonassigned. There is a trend towards higher expression within the mesenchymal stem-like subgroup.

A. TNBC (TCGA)   B. TNBC (Hess et al)   C. TNBC Subtypes

Figure: Analysis of the Relative Levels of LTBP 1-4 Expression in Different Types of Human Breast Tumors
As shown in (A) LTBP1 is more highly expressed in estrogen receptor (ER)-negative than ER-positive tumors. In contrast LTBPs 2, 3 and 4 show the opposite trend.
(B) Likewise LTBP1 is more highly expressed in basal and HER2-positive subtypes and lower in luminal subtypes. In contrast LTBP 2, 3 and 4 are more highly expressed in Luminal A subtypes.

A

B

KMplotter - RFS (red=high expression)

Breastmark - DFS (blue= high expression)

Figure 9: High LTBP2 , 3 and 4 Expression is Associated with Bette Outcome in Breast Cancer Overall.

A) Relapse-Free Survival (RFS) data were plotted using the online assessment tool Kaplan-Meier Plotter (kmplot.com) to divide breast cancer patients into high (red) and low (black) expression cohorts on the basis of best-fit criteria as indicated below the graph.
B) Disease-Free Survival (DSF) data were plotted using the online assessment tool BreastMark to divide breast cancer patients according to the median into high (blue) and low (red) expression cohorts. The results show that high LTBP2, 3 and 4 expression is correlated with better survival. Hazard Ratio (HR) and P value are as indicated.

Figure 10: High LTBP 2, 3 and 4 Expression red is Associated with Better Outcome RFS in Luminal Breast Cancer.

RFS in breast cancer patients plotted using Kaplan-Meier Plotter (kmplot.com) selecting for best-fit to divide the cohort into tumors with high (red) and low (black) LTBP 2, 3 and 4 expression. Hazard Ratio (HR) and P value are as indicated. The results show that in contrast to LTBP1, which correlates with bad outcome, high expression of LTBP 2, 3 and 4 is associated with better outcome in Luminal breast cancers.

Figure 11: High Expression of any LTBP is Associated with Worse Survival (DMFS) for HER2 Breast Cancer Patients

A) Distant Metastasis-Free Survival in breast cancer patients plotted using Kaplan-Meier Plotter (kmplot.com) selecting for best-fit to divide the cohort into tumors with high (red) and low (black) LTBP 2, 3 and 4 expression. Hazard Ratio (HR) and P value are as indicated. The results show that high LTBP2 and 4 expression has no statistically significant effect in luminal cancer whereas high LTBP3 is protective. In contrast high expression of any LTBP is associated with worse outcome for HER2 breast cancer patients.

Analysis of Breast Cancer Cell Lines: We analyzed an initial panel of breast cancer cell lines for LTBP1 mRNA levels (Figure 12). A Higher level of LTBP1 mRNA was found in Basal B (Hs578t and MDA-MB-231) and Luminal HER2-positive ER-negative cell lines (AU565 and SkBr3) compared to other Luminal and Basal A cell lines.
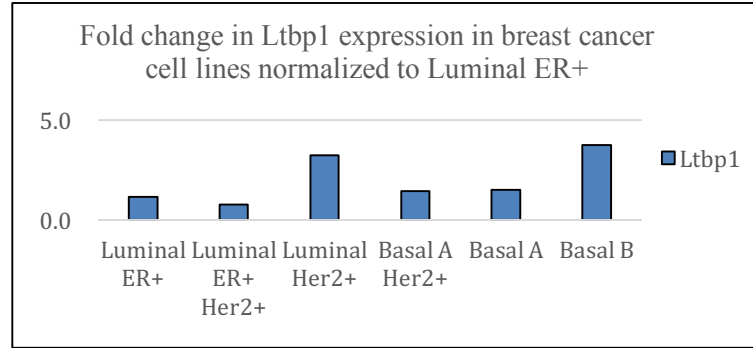

Figure 12: Cell lines were grouped into subtypes as classified by Neve et al. *LTBP1* expression was determined by qPCR normalized to levels in Luminal cell lines and averaged for each cancer subtype. *LTBP1* was expressed more highly in breast cancer cell lines classified as Basal B, HER2-positive and ER-negative which are thought to derive from breast cance rs with the worst outcome that are prevalent in young women of reproductive age.

A larger panel of human breast cancer cells lines were selected (see table below). There are only three subtypes found *in vitro* as compared to five *in vivo*. Luminal cell lines generally have an epithelial-like morphology, are non-invasive and express hormone receptors while Basal B cell lines are triple-negative and highly invasive with a more transformed morphology. *LTBP1* has two isoforms transcribed from two transcriptional start sites in the same gene a short and a long isoform (LTBP1S and LTBP1L). Importantly, whereas the bioinformatics data derived from human tumors presented in Figure 7 cannot distinguish between these isoforms as the database probes are located within the shared region of both, when analyzing cell lines, we were able to design primers that specifically detect the long or the short isoforms.

Cell Line	Subtype	Hormone Receptors
MCF7	Luminal	ER
T47D	Luminal	ER
ZR751	Luminal	ER
MDA-MB-361	Luminal	ER Her2
ZR7530	Luminal	ER Her2
AU565	Luminal	Her2
HCC1569	Basal A	Her2
HCC1954	Basal A	Her2
MDA-MB-468	Basal A	N/A
BT549	Basal B	N/A
HS578T	Basal B	N/A
MDA-MB-231	Basal B	N/A

qPCR analysis showed all cell lines expressed *LTBP1L* but on average the Luminal and Basal A subtypes showed higher expression than that of the Basal B subtype. In contrast, *LTBP1S* was highly expressed in Basal B subtypes with little to no expression detected in the Luminal and Basal A lines (Figure 13). Thus there is differential isoform expression of LTBP1 short (1S) and long (1L) isoforms in human breast cancer cell lines and LTBP1S was expressed specifically in the mesenchymal subtype of triple negative breast cancer that is associated with high motility and invasive gene signatures.
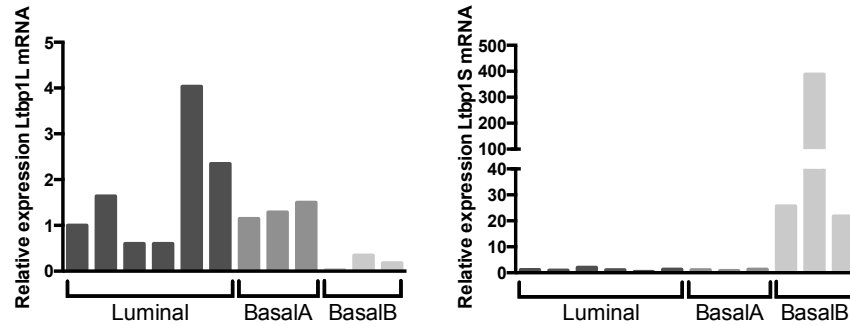

Figure 13: qPCR analysis of LTBP1L and LTBP1S expression in breast cancer cell lines. Expression is relative to first luminal sample on each graph. Data is representative of triplicate experiments.

LTBP1 expression in TNBC subtypes

To explore whether LTBP1S expression is linked to triple-negative cell lines in general or to the Basal B subtype in particular we investigated a larger panel of triple-negative breast cancer (TNBC) cell lines and performed qPCR for LTBP1S (Figure 14). TNBC cell lines have been categorized into three subtypes: Basal-like, Mesenchymal-like and Luminal Androgen Receptor. All the Basal B lines used in our initial panel were categorized as Mesenchymal-like and the one other TNBC cell line included in the panel (MDA-MB-468) is Basal-like. 4 out of the 5 Mesenchymal-like lines showed increased LTBP1S expression. LTBP1S was not seen in any others (Figure 14). This suggests that LTBP1S is specifically expressed within the Mesenchymal-like subtype of TNBC.

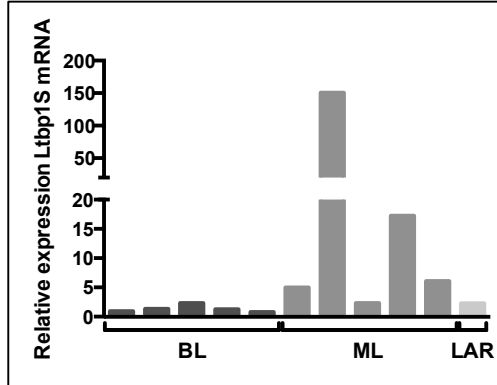| Cell Line	TNBC subtype	Traditional subtype
HCC1937	Basal-like	Basal A
HCC1143	Basal-like	Basal A
MDA-MB-468	Basal-like	Basal A
HCC38	Basal-like	Basal B
HCC1187	Basal-like	Basal A
BT549	Mesenchymal-like	Basal B
HS578T	Mesenchymal-like	Basal B
SUM159PT	Mesenchymal-like	Basal B
MDA-MB-231	Mesenchymal-like	Basal B
MDA-MB-157	Mesenchymal-like	Basal B
MDA-MB-157	LAR	Luminal

Figure 14: LTBP1S expression in triple-negative breast cancer cell line panel. BL: Basal-like, ML: Mesenchymal-like, LAR: Luminal Androgen Receptor. Data representative of three repeats.

LTBP protein expression

In an attempt to detect LTBP1 protein expression we tested two antibodies, Ab39 and LIC, on frozen and paraffin embedded PFA-fixed normal breast samples derived from discarded reduction mammoplasty. Both antibodies localized LTBP1 in close proximity to the basal layer of human TDLU, similar to our findings in mice of LTBP1 encasing the mammary ducts. Both LTBP1 antibodies worked best on frozen sections. We concurrently investigated the binding partner for LTBP1 by performing co-immunofluorescence for fibrillin 1 and elastin. Elastin antibodies proved to be specific for mouse and did not stain human tissue. The fibrillin antibody worked well on human tissues specimens and showed robust expression next to the LTBP staining. However, staining with the LTBP1 antibodies on archival tissues formalin-fixed for long periods and paraffin-embedded specimens was poor and were unable to get specific staining despite optimizing for a variety of epitope retrieval conditions in these samples. We continue to focus effort on solving issues concerning antigen retrieval to allow examination of LTBP1 expression in archival human breast cancers.
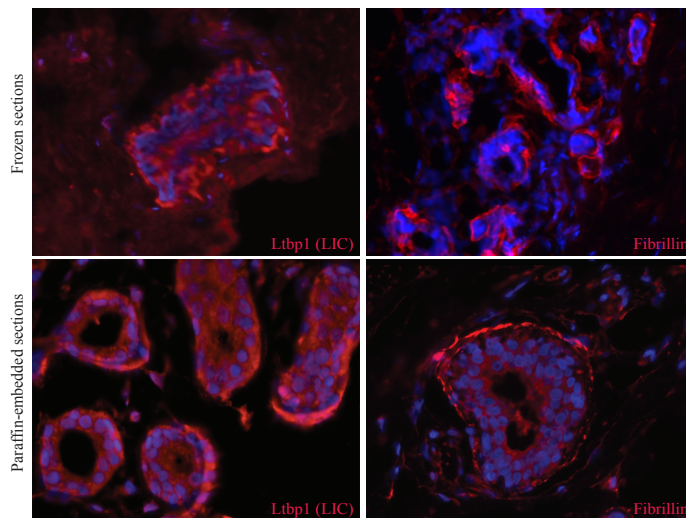

Figure 15: Immunofluorescent detection of LTBP1 with LIC antibody (left panels) and Fibrillin (right panels) in frozen (Top) and formalin-fixed paraffin embedded (bottom) sections**.**

Conclusions: In conclusion these analyses show that *LTBP1* expression correlated with absence of estrogen receptor and is associated with poor prognosis in breast cancer, particularly within Basal and Her2 subtypes. It is highest within TNBCs, particularly those of the most aggressive MSL subtype. These findings support the concept that LTBP1 is a prometastatic factor. Given our previous finding of isoform specific expression of LTBP1S in TNBC cell lines it will be important to raise specific antibodies capable of examining if LTBP1S expression correlates with specific TNBC tumor subtypes and survival. In contrast other LTBPs are more highly expressed in luminal A estrogen receptor-positive tumors and their high expression in this type of breast cancer is associated with better outcome. Higher expression of any LTBP is associated with worse outcome within the HER2 subtype of breast cancer.

Task 2: Determine the consequences of modulating LTBP1 level in breast cancer cell lines on motility, invasion and metastasis

a) *LTBP1* expression in highly metastatic variants of breast cancer cell lines.
To test the hypothesis that LTBP1 is a prometastatic factor we examined *LTBP1* expression in two metastatic variants of the MDA-MB-231 cell line. These were selected for increased ability to form lung (231-LM2) and brain (231-AdM) metastasis compared to the parental line. The brain metastatic line was recently re-assigned as an adrenal metastasis line. These lines together with their parental cell line were purchased from Dr. Juan Massague (Memorial Sloan Kettering Cancer Center) and analyzed by qPCR for total LTBP1, LTBP1S and LTBP1L. Both metastatic variants showed increased expression of LTBP1L and LTBP1S was up-regulated within the LM2 line (Figure 16). A qPCR probe that detects both isoforms recapitulates that of LTBP1S suggesting that LTBP1S is the major isoform expressed in MDA-MB-231 cells. This analysis supports our hypothesis by showing a correlation between increased LTBP expression and enhanced metastasis and established that MDA-MB-231 cells and their metastatic variant LM2 provide a good in vitro model in subsequent experiments for testing the effect of downregulating LTBP1 expression on migration and invasion.

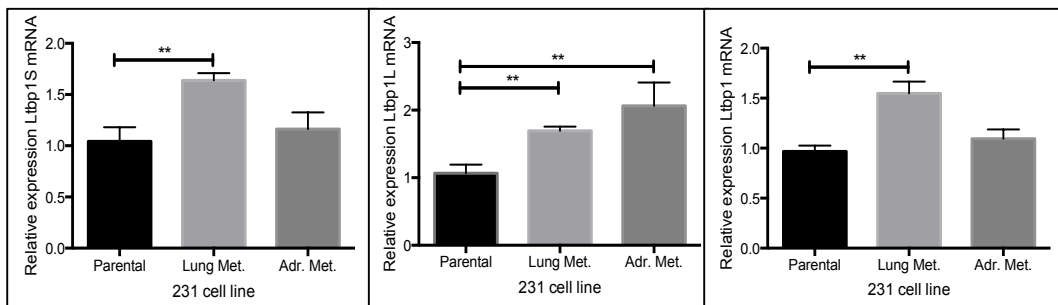

Figure 16: LTBP1 expression in MDA-MB-231 parental line and lung and adrenal metastatic variants. LTBP1S (left), LTBP1L (center) and total LTBP1 (right) mRNA was measured by qPCR. Data is presented relative to parental line. ** t-test p<0.01

b) Generation of an inducible lentiviral LTBP1 knock down system.

We cloned two LTBP1 hairpins (sh1 within the coding region, sh2 in the 3'UTR and scrambled controls) into the lentiviral vector pLKO-GFP and pLKO-RFP respectively and tested their effect in MCF10A cells, which express LTBP1 robustly. Both hairpins efficiently knocked down LTBP1 mRNA as assessed by RT-PCR (Figure 17 bottom panels) and induced significant detachment of cells and cell death.
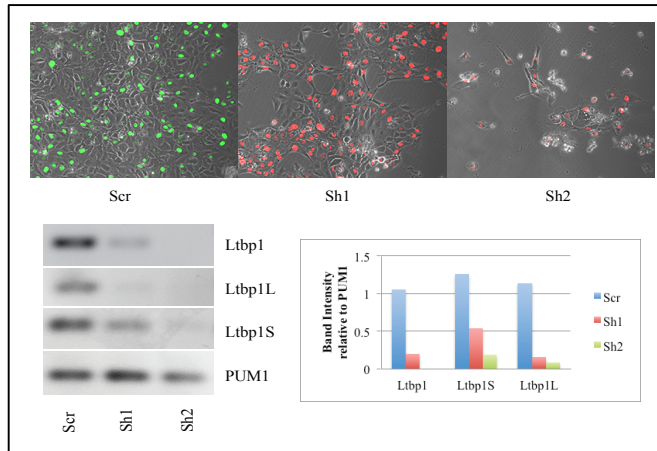

Figure 17: Top panel - MCF10A cells infected with control shRNA hairpin (Scr) or LTBP1 shRNA hairpin (Sh1 and sh2). sh2 induces cell rounding and detachment. Bottom left shows RT-PCR detection of LTBP1 expression in control (Scr) infected cells and efficient knock down of LTBP1L by sh1 and both isoforms of LTBP1 by sh2. Bottom right panel shows the data for LTBP1 mRNA expression normalized to expression of control PUM1.

To circumvent this issue we recloned the hairpins and Scr control into an inducible system, pLKO Tet-on puro (Addgene 21915), which allows expression of the hairpins to be induced by the addition of doxycycline to the media. It also confers puromycin resistance to allow selection of a pure population after infection with the vector. We infected MDA-MB-231-LM2 cells with these inducible vectors, selected with puromycin and induced knockdown by adding doxycycline to the cells every 2 days for 5 days. Knockdown was assessed by PCR. Efficient knockdown of LTBP1 was observed within 24 hours and maintained throughout the time course examined. Knockdown of LTBP1L and 1S was confirmed (Figure 18). No morphological changes or cell death was observed (Figure 19).
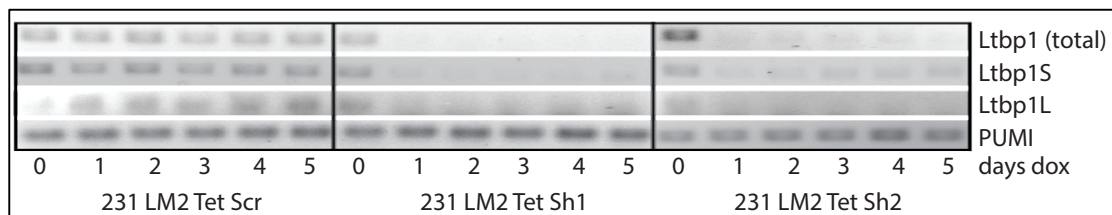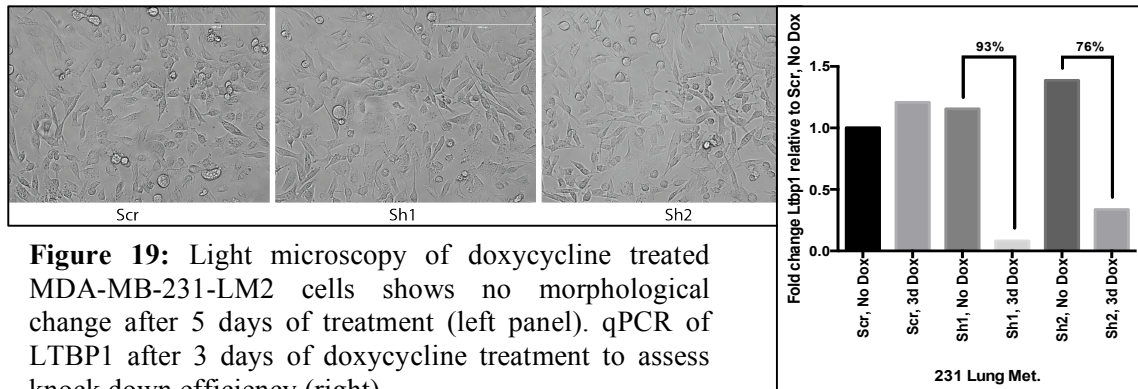

Figure 18: PCR of LTBP1 in a 5-day time course of doxycycline treated MDA-MB-231-LM2 cells containing Scr or LTBP1 knockdown vectors to show efficient knockdown is maintained.

Figure 19: Light microscopy of doxycycline treated MDA-MB-231-LM2 cells shows no morphological change after 5 days of treatment (left panel). qPCR of LTBP1 after 3 days of doxycycline treatment to assess knock down efficiency (right)

c) Effects of LTBP knock down on cell proliferation

Sh1 showed the strongest knockdown though both are over 75% reduced (Figure 6) but all results were confirmed to also be true for Sh2 eliminating concerns of off-target effects. Before using these knockdown cells to assess changes in adhesion and motility it was important to establish if proliferation was changed. Proliferation was assessed over 4 days and no change in cell division was found between knockdown and control cells (Figure 20).

Figure 20: Cell growth of mDA-MB-231-LM2 cells over 4 days after 3 days of doxycycline treatment. Cells were counted using hemocytometer.

d) Effects of LTBP1 knock on cell adhesion

To assess the effects of LTBP1 knockdown on cell adhesion, MDA-MB-231-LM2 cells were treated with doxycycline for 3 days. 50,000 cells were plated per well of a 24 well plate, which was coated with Fibronectin or left either uncoated. After an hour non-adherent cells were removed by washing and attached cells were fixed, stained and counted. No difference in adhesion was observed on uncoated plastic. There is a minimal increase in adhesion onto Fibronectin (Figure 21).

Figure 21: Number of cells adhered to uncoated or Fibronectin coated plastic after 1 hour. Three fields of view were counted per well and each conditioned was performed in triplicate (* p<0.05).

To assess changes in migratory and invasive behavior of LTBP1 knockdown in MDA-MB-231-LM2 cells we first performed scratch assays. Cells were grown in the presence of doxycycline for 3 days to confluence. A pipette tip was then used to create a wound through the cells. Cells were imaged every 4 hours to calculate wound closure rate. No difference in the wound closure rate was observed between the cells lines suggesting that knockdown of LTBP1 has no effect on MDA-MB-231-LM2 cell migration *in vitro* (Figure 22).
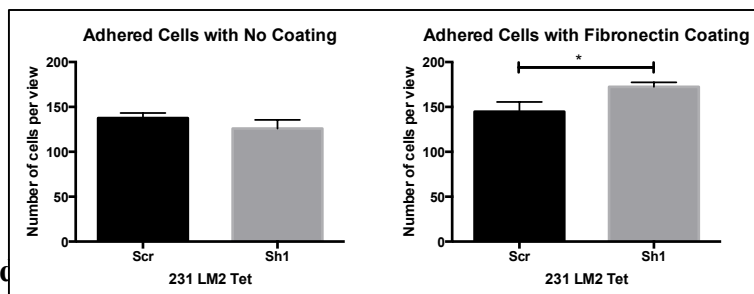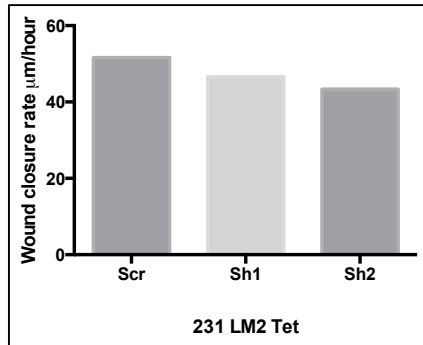

Figure 22: Closure rate of an artificially introduced wound. Size of remaining gap was measured over 8 hours and the closure rate calculated. Results represent three independent experiments. No significant difference.

However, MDA-MB-231-LM2 cells are not epithelial in morphology making scratch assays difficult to evaluate as they do not migrate as a sheet but as single cells, some of which bridge the wound very quickly while others hardly move. Therefore, we sought to investigate this further by using a transwell assay. Cells treated with doxycycline for 3 days were plated in low serum conditions above a well with normal serum and allowed to migrate for 3 hours through the transwell and then collected and stained. Again no difference was observed confirming that knockdown of LTBP1 does not affect migration (Figure 23).

Figure 23: Transwell migration assay. Number of cells passing through a transwell in response to a chemoattractant gradient over 3 hours. Three fields of view were counted per well and each condition was performed in triplicate. Results representative of three independent experiments. No significant difference.

e) Effects on cell invasion

To examine if LTBP1 knockdown affects the invasive behavior of these cells we assayed transwells coated with matrigel (Figure 24). Cells were plated in low serum above the matrigel and with high serum below. After 18 hours cells that had invaded through the matrigel were fixed, stained and counted. Knockdown of LTBP1 lowered the invasion efficiency of MDA-MB-231-LM2 cells (Figure 24). This is consistent with the bioinformatics data present in Aim 1 showing LTBP1 is associated with poor prognosis, which in turn is due to invasive cancer.

Figure 24: Invasion assay. MDA-MB-231-LM2 cells were plated into transwells coated with matrigel and allowed to invade over 18 hours. Cells were stained and counted. Three fields of view per well were counted and each condition was run in triplicate. Result representative of three independent experiments (** p<0.01).

In conclusion cell proliferation, adhesion and migration remained unaltered but the ability of cells lacking LTBP1 expression to invade through matrigel in a transwell assay was severely compromised. This supports the concept that LTBP1 is proinvasive.

f) Expression of LTBP binding partners

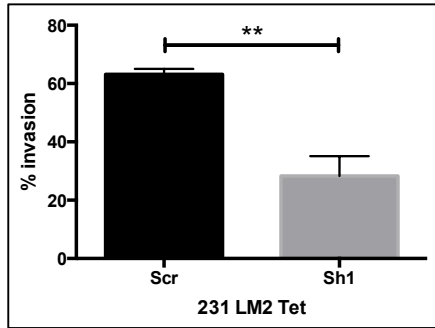Ltbp1 can interact with small latent complex of three isoforms of Tgf-beta and after secretion it binds to Fibronectin (Fn1) and Fibrillin 1 (Fbn1). To elucidate if breast cancer cells produce these binding partners we investigated a panel of breast cancer cell lines including MDA-MB-231 PCR for their mRNA expression of TGF-beta ligands extracellular matrix binding partners and TGF-beta receptors (Figure 25). Luminal type breast cancer cell lines expressed Tgf-beta3 whereas Basal A and Basal B cell lines express Tgf-beta1 and Tgf-beta2 (Figure 25a). Fibronectin was detected more broadly across the panel but was strongly expressed in the Basal A and Basal B lines but only Basal B cell lines expressed Fibrillin 1 (Figure 25b). This suggests that these more highly invasive breast cancer cell lines. To further query the correlation we performed PCR in the triple-negative breast cancer cell line panel (Figure 25c). All 5 Mesenchymal-like lines expressed both Fbn1 and Fn1 while only 2 out of 5 Basal-like cells show expression. This shows that Mesenchymal-like TNBC cells are able to build their own cancer stem cell niche, supporting the concept that the seed of breast cancer generates its own prometastatic soil. Luminal cells express predominantly TGF-beta receptor 1 whereas Basal cells express TGF-beta receptor 2 and Basal B cell lines predominantly express TGF-beta receptor 3 (Figure 25d).

Collectively these data suggest that only Basal B cells generate a fibrillary matrix that will permit presentation and integrin-mediated stretch activation of TGF-beta and that they have the necessary receptors to respond.

g) In vivo metastasis assays. To test the ability of LTBP1 to facilitate metastasis we performed a pilot experiment introducing MDA-MB-231 cells harboring doxycycline-inducible sh-scr and sh-ltbp1 hairpins into the mammary fatpads of immunocompromised mice (Figure 26). Seeding of the primary tumors was successful. Once a suitable replacement for Dr Chandramouli can be hired we will repeat these experiments with larger cohorts of animals and follow the spread of cells by IVIS to bring this project to publication.

Figure 2 : Breast Cancer Cell line Expression of LTBP1 ECM TGF-beta Partners and Receptors
PCR analysis of the mRNA expression of the potential binding partners of LTBPs in different subtypes of breast cancer cell lines
(Luminal   MCF ,T47D, ZR751, MDA-MB-361, ZR7530, AU565) (Basal A= HCC159,   HCC1954, MDA-MB-468) and (Basal B   BT549,   HS578T, MDA-MB-231) as indicated. Gapd serves as a cDNA control.

a) TGF-beta ligands (TgfB1, TgfB2, TgfB3). Note TgfB2 in Basal B and TgfB3 in luminal cell types
b) The extracellular matrix partners Fibrillin1 (Fbn1) and Fibronectin (Fn1)
c) Fibrillin and Fibronectin expression in different TNBC subtypes as indicated. Note Basal B and the MSL subtype of TNBC cell lines express both   CM binding partners.
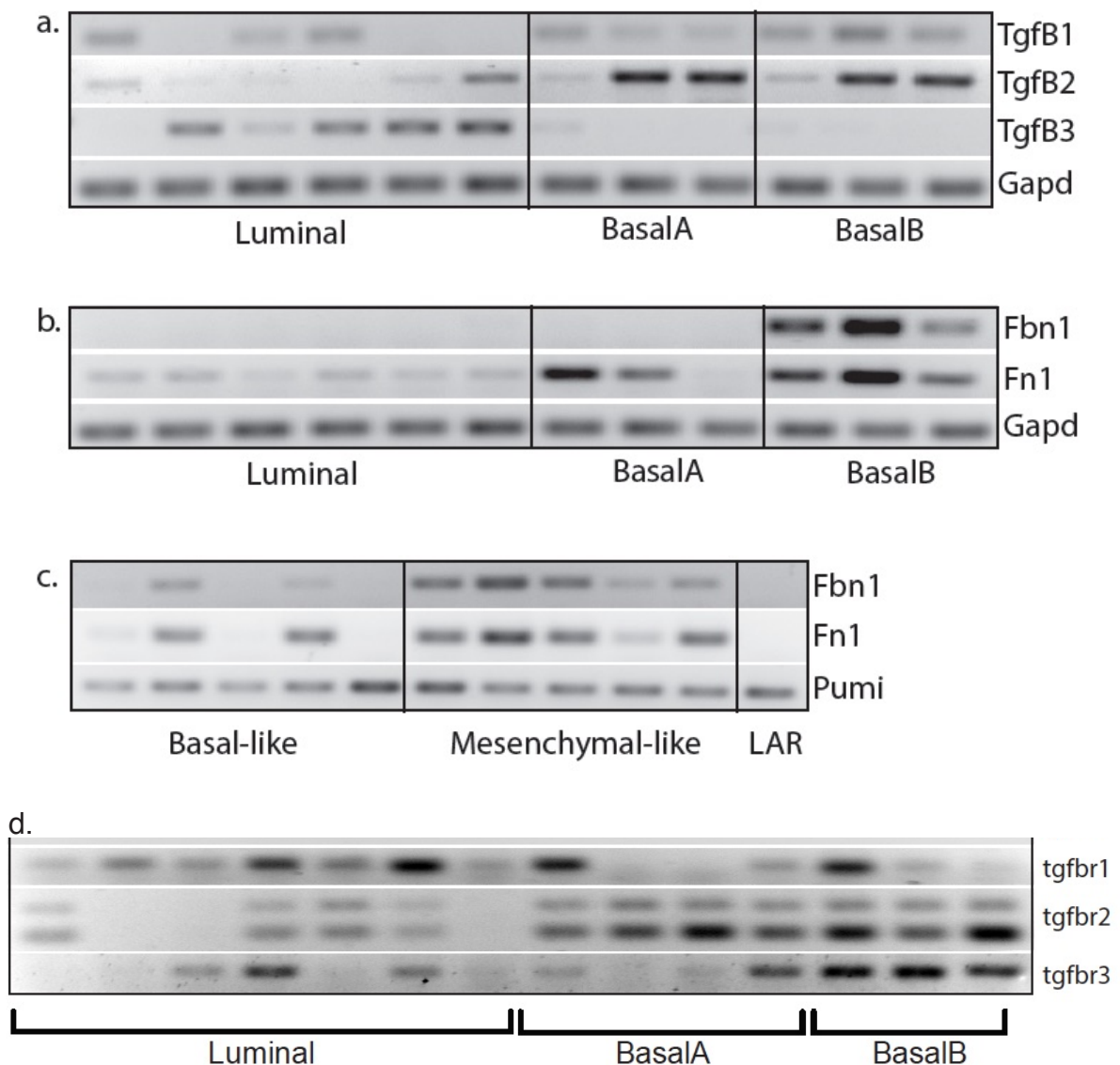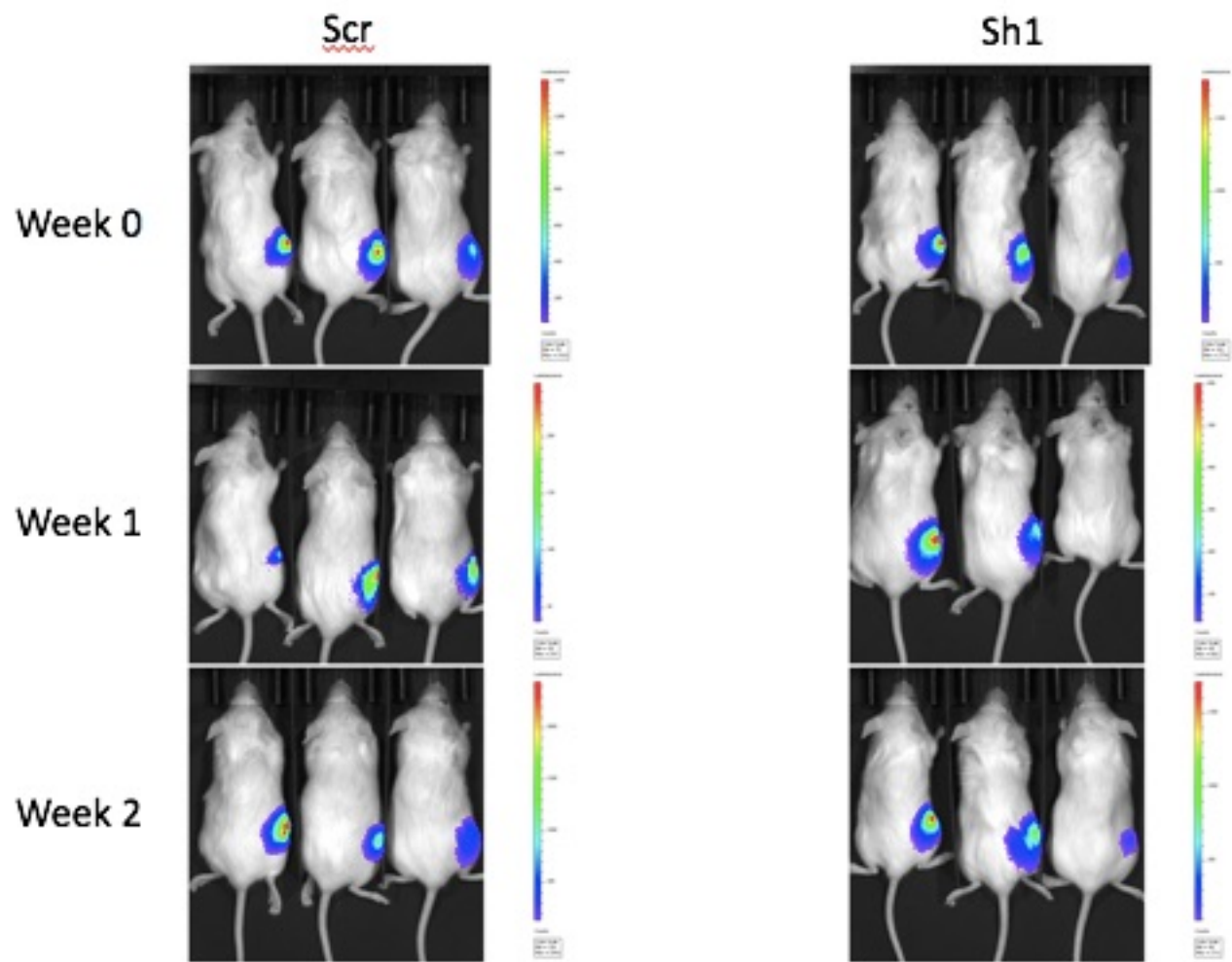d) TGF-beta receptor expression (Tgfbr1, 2, 3).

Figure 26: Metastasis Assay for TNBC Cell Lines with Lentiviral Ltbp1 Knock-down

MDA-MB-231 cells were infected with lentivirus driving expression of sh-scr or sh-ltbp1 hairpins together with a luciferase cassette then injected into the mammary fatpads of SCID recipients. The cells were detected by in vivo imaging for luciferase expression (IVIS).
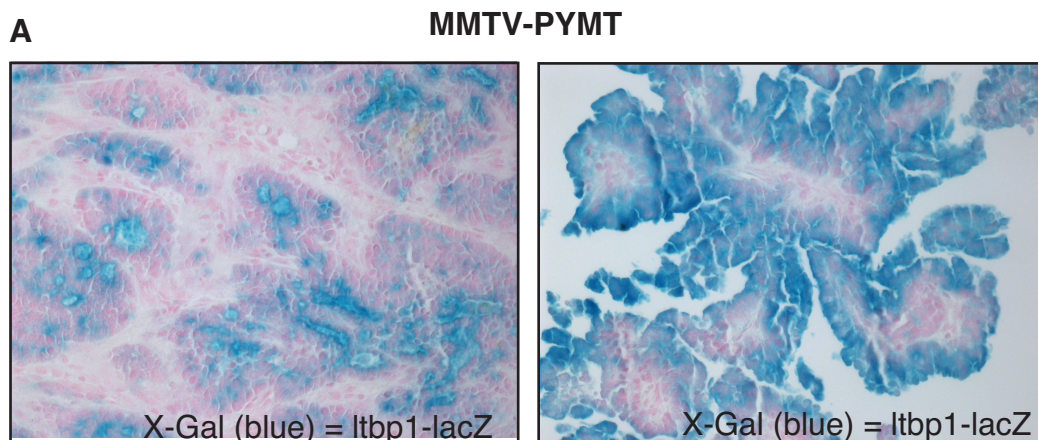
Task 3: Determine the pathological significance of gain and loss of LTBP1 in mice (75% complete).

As we reported previously we encountered significant problems in cloning human LTBP1 into pHIV-ZsGreen lentiviral vector due to the large size of the insert and similarly in constructing a transgene to permit overexpression of LTBP1. In the mean time we have checked for expression of *Ltbp1* I the PYMT tumors by crossing these mice to a reporter strain *Ltbp1-lacZ* where beta-galactosidase is produced under the control of the Ltbp1 promoter. Intriguingly, Ltbp1 is seen at the pushing margins of highly invasive segments of these tumors (Figure 27b). We have harvested primary tumors from PYMT mice and attempted to infect primary MECS with lentiviral knock down shRNA hairpins to determine the effects of modulating LTBP1 on tumor progression. We have also determined the mRNA expression levels of all LTBPs within MMTV-Wnt1, MMTV-neu, MMTV-PYMT, C3(1)-Tag compared to normal FVBN controls (Figure. 27a). Intriguingly, *Ltbp1* is elevated in the metastatic C3(1)-Tag and MMTV-PYMT strains and downregulated in the poorly metastatic MMTV-neu strain. *Ltbp4* is markedly downregulated in all tumors. *Ltbp2* and *Ltbp3* are modestly increased. From these analyses we conclude that C3(1)-Tag model would be a good model to explore the role of *Ltbp1* in tumor progression. This mouse strain resembles human DCIS and 20% of mice progress to show a similar pattern of metastasis to that seen in humans.

Fig. 27. Expression of Ltbp1-4 in Mouse Mammary Tumor Models

(A) Ltbp1-lacZ reporter expression detected by X-Gal staining (blue) in sections of a MMTV-PYMT tumor. Note the luminal expression within differentiated regions (left panel) and expression on the pushing margins of highly invasive regions (right panel)

(B) qRT-PCR analysis of Ltbp1 (blue), Ltbp2 (red), Ltbp3 (green), and Ltbp4 (purple) mRNA expression in four different transgenic mouse mammary tumor models: MMTV-Wnt1, MMTV-neu, MMTV-PYMT,C3(1)-Tag compared to normal FVBN controls. Note Ltbp4 is downregulated in all tumors; Ltbp1 is elevated in the metastatic C3(1)-Tag and MMTV-PYMT strains and downregulated in the poorly metastatic MMTV-neu strain.

A

MMTV-PYMT

X-Gal (blue) = ltbp1-lacZ   X-Gal (blue) = ltbp1-lacZ

B

FVBN control   MMTV-Wnt1

FVBN control   MMTV-PYMT

FVBN control   C3(1)-Tag

FVBN control   MMTV-neu

- ➤ **What opportunities for training and professional development has the project provided.**
- ➤ **Year 1**
- ➤ Dr. Catina Crismale Gann took a course in "Translating Cancer Discovery into Clinical Practice" and received individual mentoring on mammary stem cells, acquired skills in mammary epithelial cell preparation, FACS analysis, Lentiviral construction and transplantation.
- ➤ **Year 2**
- ➤ Dr. Gann took a course in "Scientific Ethics" and acquired skills in qPCR, and FACS analysis.
- ➤ Dr. Oliver acquired skills in assessing Lentiviral knock down and obtained a fellowship from The Susan G Komen Foundation and formed and met with two members of her committee who are both scientists and breast cancer survivors

  Year 3

  Dr. Chandramouli acquired skills in shRNA Lentiviral knock down, survival surgery and mammary transplantation and IVIS tracking of breast cancer cell dissemination.

- ➤ **How were the results disseminated to communities of interest**
- ➤ Dr. Cowin has presented this work in two seminars at Rutgers Department of Animal Science and at UMDNJ/Rutgers Cancer Center.
- ➤ Dr. Cowin presented this work to incoming graduate student candidates of the Pharmacology training program of the NYU Sackler Graduate Institute in 2014 and 2015.
- ➤ Dr. Oliver presented to the NYU Developmental Genetics and Stem Cell Work in Progress meeting in 2014.
- ➤ Dr. Cowin presented this work to the Developmental Biology Program, Institute of Biotechnology, Helsinki, Finland on Feb 12 2015.
- ➤ Dr. Cowin presented this work to the Skirball Institute Faculty Works in Progress bimonthly meeting 2015 and 2016.
- ➤ Dr. Gann presented to the NYU Molecular Pathology and Immunology Works in Progress meeting in 2015.
- ➤ Dr. Oliver presented this work at the GRC on Cell Contact and Adhesion, Proctor Academy, NH, June 2015
- ➤ This work was presented to our scientist breast cancer survivor advocates at the GRC meeting above: Dr. Cara Gottardi, Northwestern University and Dr. Juliet Daniels, McMaster University in June 2015.
- ➤ Dr. Cowin presented this work at the GRC on Mammary Gland Biology June 2016
- ➤ Dr. Cowin presented this work in an invited seminar at The University of Georgia in Feb 2017
- ➤ Dr. Cowin presented this work in an invited seminar to the Department of Veterinary Medicine, Cambridge, UK in June 2017
- ➤ Dr. Cowin presented this work at the Gordon Conference on Elastin, held in Portland, Maine in July 2017
- ➤ Dr. Cowin presented this work to the Department of Matrix Biology and Regenerative Medicine, University of Manchester, UK in Sept 2017

➤ **What do you plan to accomplish during the next reporting period to accomplish the goals and objectives. (**N/A final report). I am continuing to work on completing the in vivo metastasis experiments with LTBP1 deficient cells to bring this work to publication.

4. IMPACT

➤ **What was the impact on the development of the principal discipline of the project.**

➤ Our results have shown that the breast tumor suppressor and metastasis promoter TGFbeta are spatially and temporally restricted in a very dynamic spatio-temporal pattern during breast development and not, as previously thought, ubiquitously available to all mammary cells. This means TGF-beta signaling in responding cells is very tightly controlled by LTBP1 expression. Our data reveal that fibrillin and elastin are expressed at significant levels within breast stroma. As there are no other studies on LTBP1 or fibrillin in breast this has opened up a new field and expanded our understanding of the stromal composition of the breast.

➤ Our results have shown that high levels of LTBP1L occur in aggressive forms of basal and HER2 positive breast cancer, and that higher levels identify patients with particularly poor outcome within these groups. The unpublished data reported herein introduce the concept that LTBP1 expression has value as a prognostic indicator of patient outcome in HER2 and Basal type breast cancer. This has pioneered a new field in breast cancer research since there are no studies besides our own on LTBP1.

➤ LTBP1S is highly expressed within the mesenchymal-like subset of TNBC cell lines and these cells appear to be the only forms of breast cancer that has all the apparatus to connect LTBP-TGF-beta complex to the ECM, activate and respond to it.

➤ Our unpublished data reported herein suggest that other LTBP family members are associated with good outcome and therefore it is likely of significance that LTBP4 is downregulated in all murine breast cancer models and that LTBP1 is upregulated in metastatic models but downregulated in benign models. Our in vitro studies support the concept that LTBP1 aids cell invasion.

➤ **What was the impact on other disciplines**
➤ Our in vitro studies support the concept that LTBP1 is proinvasive in breast. As LTBP1 is expressed in many other tissues these findings could also have relevance for many types of carcinoma.
➤ Our work has illuminated potential roles for LTBP1 in the physiology of lactation particularly during developmental periods of intense autophagy during breast involution. This opens the possibility that LTBP1 serves as a niche factor essential for maintaining stem cell viability during the extensive cell death of the surrounding differentiated epithelium. The expression pattern of LTBP1 suggests it may play a critical role in regulating epithelial stem cell quiescence and survival that could be relevant in many epithelial tissues and cancers.

➤ **What was the impact on technology transfer**
➤ Nothing to report

➤ **What was the impact on society beyond science and technology**
➤ Linking breast development factor to breast cancer risk opens the door to preventative strategies linked to reproductive history.

5. CHANGES/PROBLEMS

➤ **Delays in approach and reasons for delay**

➤ **Actual anticipated problems or delays and actions or plans to resolve them**
Problems with retrieving antigens in archival tissue fixed for long periods prevented detection of protein with antibodies therefore we focused on in silico and RNA analyses of LTBP expression. We were unable to construct the LTBP1 transgenic cassette due to problems with the length and also the high GC content of the cDNA.

➤ **Changes that had a significant impact on expenditures –** Dr. Oliver departed prematurely from the project due to relocation for family reasons resulting in a surplus at the end of year 3. This was rebudgeted as a no cost extension to support Dr. Chandramouli, a research associate skilled in transplantation techniques, to complete the experiments. NYU administration insisted on terminating Dr. Chandramouli's position in December in anticipation of her severance entitlement and it was impossible to hire another person with sufficient skills to complete the experiments. As Dr. Chandramouli declined severance payment in favor of securing a position in another NYU laboratory the remaining salary support that would have paid her has been returned.

➤ **Significant changes in use or care of human subjects, animals, biohazards or select agents** - Nothing to report

6. PRODUCTS
➤ **Publications, conference papers and presentations**
- *Manuscripts: See attached Chandramouli et al*

- We are writing up the work detailed above to be submitted as a manuscript to Breast Cancer Research.

- *Books etc:* Nothing to report

- *Other publications, conference papers and presentations*
Dr. Cowin presented this work at the GRC on Mammary Gland Biology, June 2016. Dr. Cowin presented this work at the Gordon Conference on Elastin, held in Portland, Maine in July 2017
Acknowledgement of Federal Support: YES
➤ **Website(s) or other internet site (s):** Nothing to report

- ➢ **Technologies or Techniques:** Nothing to report

- ➢ **Inventions, patent applications and/or licenses:** Nothing to report

- ➢ **Other products:**
   - o **Research material:** Generation of inducible lentiviral LTBP1, LTBP1L and LTBP1S hairpin constructs

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS
Individuals working on the project:

Name	Pamela Cowin
Project Role	P.I.
Research Identifier	
Nearest person month worked	12
Contribution to project	Directed research
Funding Support	DOD BC123572 20%

Name	Anupama Chandramouli
Project Role	Postdoctoral fellow
Research Identifier	
Nearest person month worked	6
Contribution to project	Performed work on Aim 3
Funding Support	None

Name	Carrie Oliver
Project Role	Postdoctoral fellow
Research Identifier	
Nearest person month worked	3
Contribution to project	Performed work on Aim 2
Funding Support	Susan G Komen Fellowship 100%

Name	Catina Crismale Gann
Project Role	Postdoctoral fellow
Research Identifier	
Nearest person month worked	3
Contribution to project	Performed work on Aim 1
Funding Support	DOD BC123572 100%

- ➢ **Has there been a change in the active support of the PI or senior key personnel since the last reporting period**

P.I. Dr. Pamela Cowin – No change

Postdoctoral Fellow – Dr. Catina Crismale Gann and Carrie Oliver were replaced by Dr. Chandramouli. Dr. Chandramouli's position ended in December 2016.
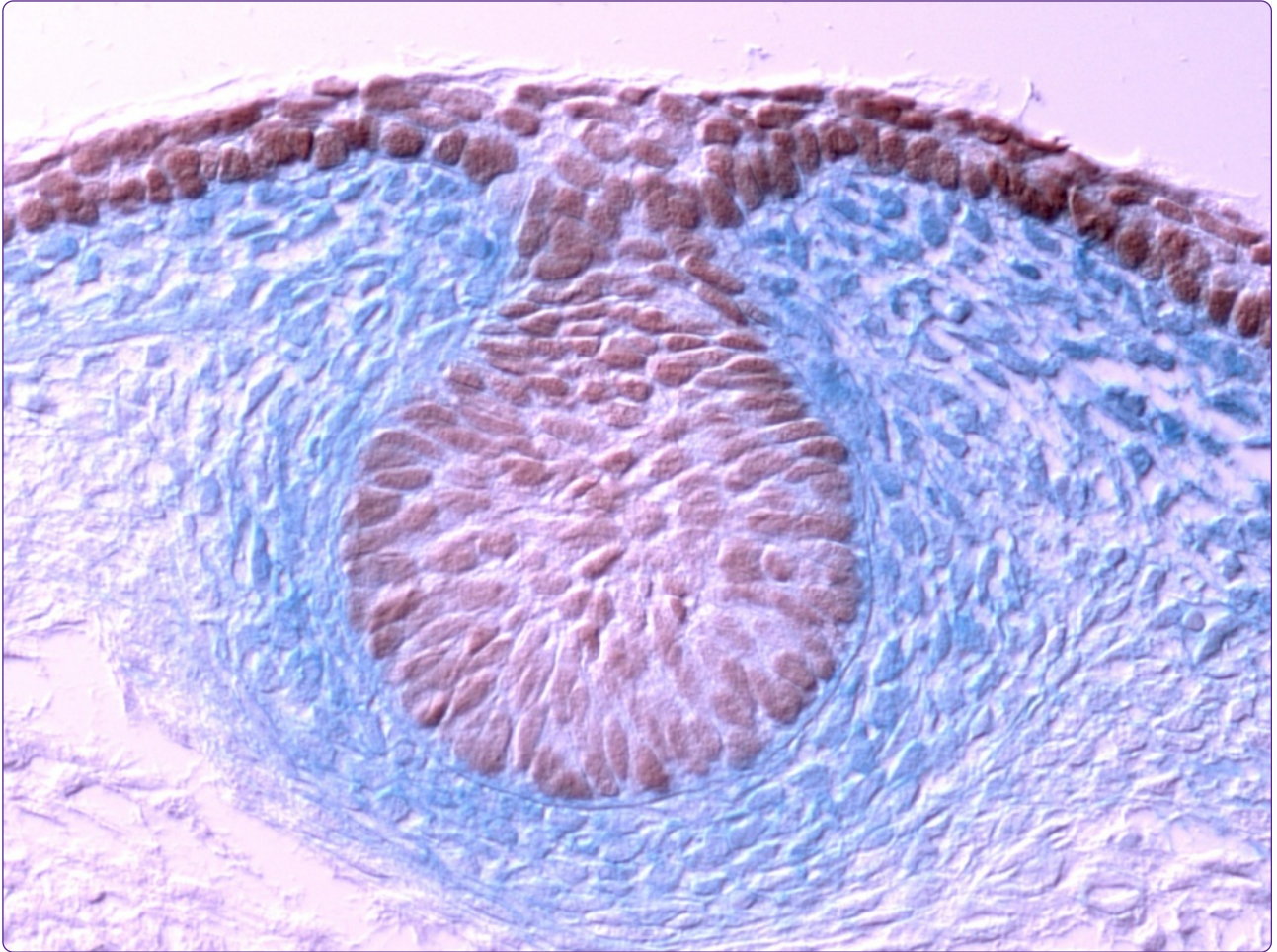
> **What other organizations were involved as partners?**

None

8.   SPECIAL REPORTING REQUIREMENTS N/A

9.   APPENDICES

Chandramouli et al 2013

Ltbp1L is focally induced in embryonic mammary mesenchyme, demarcates the ductal luminal lineage and is upregulated during involution

Chandramouli *et al.*

Breast Cancer
RESEARCH

RESEARCH ARTICLE	Open Access

Ltbp1L is focally induced in embryonic mammary mesenchyme, demarcates the ductal luminal lineage and is upregulated during involution

Anupama Chandramouli[1,2†], Julia Simundza[1,2†], Alicia Pinderhughes[1†], Minoti Hiremath[1], Gustavo Droguett[3], David Frendewey[3] and Pamela Cowin[1,2*]

Abstract

Introduction: Latent TGFβ binding proteins (LTBPs) govern TGFβ presentation and activation and are important for elastogenesis. Although TGFβ is well-known as a tumor suppressor and metastasis promoter, and LTBP1 is elevated in two distinct breast cancer metastasis signatures, LTBPs have not been studied in the normal mammary gland.

Methods: To address this we have examined *Ltbp1* promoter activity throughout mammary development using an Ltbp1L-LacZ reporter as well as expression of both Ltbp1L and 1S mRNA and protein by qRT-PCR, immunofluorescence and flow cytometry.

Results: Our data show that *Ltbp1L* is transcribed coincident with lumen formation, providing a rare marker distinguishing ductal from alveolar luminal lineages. *Ltbp1L* and *Ltbp1S* are silent during lactation but robustly induced during involution, peaking at the stage when the remodeling process becomes irreversible. *Ltbp1L* is also induced within the embryonic mammary mesenchyme and maintained within nipple smooth muscle cells and myofibroblasts. Ltbp1 protein exclusively ensheaths ducts and side branches.

Conclusions: These data show *Ltbp1* is transcriptionally regulated in a dynamic manner that is likely to impose significant spatial restriction on TGFβ bioavailability during mammary development. We hypothesize that Ltbp1 functions in a mechanosensory capacity to establish and maintain ductal luminal cell fate, support and detect ductal distension, trigger irreversible involution, and facilitate nipple sphincter function.

Introduction

Latent transforming growth factor β (TGFβ) binding proteins (LTBPs) are regulators of elastogenesis and TGFβ [1]. Their critical role in tissue development, homeostasis and resilience is demonstrated by the fact that *LTBP* loss-of-function mutations underpin a growing list of human genetic syndromes [2-4]. Gain of *LTBP* gene expression also has pathological consequences: *LTBP1* is upregulated in two breast cancer metastasis signatures and is one of only six genes found in common to both [5,6].

Ltbp genes encode a family of secreted proteins, Ltbp1-4, that show extensive sequence homology to fibrillins, which polymerize to form microfibrils and coat elastic fibers [1,7]. Ltbp proteins are initially deposited onto fibronectin and later transferred to microfibrils by interaction with fibrillins [8]. Their importance for the structural integrity and tensile function of the extracellular matrix (ECM) is illustrated by the pathologies seen in *Ltbp4S*-null mice resulting from defective elastic-fiber formation in the intestine, lung and pulmonary artery and in humans with Urban-Rifkin-Davis syndrome [4,9,10].

In addition to their contribution to ECM structure, Ltbp1, Ltbp3 and to a lesser extent Ltbp4 govern the spatial patterning and activation of TGFβ. TGFβs are secreted in a latent form, encapsulated by their cleaved latency-associated propeptide (LAP), and deposited within the ECM for subsequent activation. Ltbps post-

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translationally regulate TGFβ in three ways. First, they chaperone the association of TGFβ with LAP and through preferential binding affinities control which of three TGFβ isoforms emerge from the cell [11]. Second, Ltbps incorporate latent TGFβ within the ECM thereby determining where TGFβ is presented to its receptors [12]. Third, Ltbps provide a key link between the ECM and the cell surface that is essential for stretch activation of TGFβ [13-15]. Both integrins and Ltbp bind to LAP. Thus, when Ltbp1 is anchored in a stiff ECM and stress fibers exert tension on integrins, conformational changes occur in LAP that lead to release of the active TGFβ [13,14,16]. One major response to TGFβ signaling is synthesis of new matrix proteins [17]. Thus, Ltbps create a mechanosensory system that generates a highly localized feedback response to cell traction or tension within the microenvironment [1,18].

Mouse mutants have illuminated the roles of Ltbps in tissue homeostasis and their involvement in human pathology. *Ltbp1* hypomorphs show facial dysmorphia [19] and *Ltbp1L* loss leads to embryonic lethality due to heart malformation [20], *Ltbp2* loss-of-function mutations cause glaucoma in humans and lens defects in mice [21], *Ltbp3* loss-of-function mutation results in severe bone malformation [3,22,23] and *Ltbp4S*-null mice show multiple organ defects [4,9,10]. In some mutants the prevailing pathology reflects compromised elastogenesis [10,24]. In others the phenotype can be ameliorated by concurrent deletion or pharmacological antagonism of TGFβ, supporting the central role of Ltbps in TGFβ biology and pathology [10].

Three TGFβ isoforms are differentially expressed and exert multiple effects during mammary development [25]. Loss- and gain-of-function studies have shown that TGFβ signaling restrains pubertal ductal extension and side branching by stimulating Wnt5a expression [26-31]. TGFβ1 influences stem cell regenerative potency and cell-fate determination and has been proposed to suppress precocious alveologenesis in the adult gland prior to pregnancy [27,32-36]. Weaning massively induces TGFβ3 expression, and this surge is essential for the demise of the differentiated glandular epithelium and remodeling events during mammary involution [37,38]. TGFβ1 has also been the object of intense investigation due to its pathological relevance for breast cancer [39,40] where it acts as a tumor suppressor in premalignant lesions and at later stages promotes metastasis through induction of epithelial-to-mesenchymal transition (EMT).

Knowledge of Ltbp's temporal and spatial expression pattern is central to understanding TGFβ signaling both in the physiological setting of the normal mammary gland and in breast cancer. Yet to date there have been no studies on Ltbp within the normal mammary gland.

Here we show that *Ltbp1* is induced in a highly specific temporal and spatial pattern throughout mammary development, supporting the concept that dynamic transcriptional regulation of *Ltbp1* provides a mechanism to impose considerable restriction on TGFβ bioavailability. *Ltbp1L* is upregulated early during embryonic mammary mesenchyme specification and is sustained in smooth muscles of the nipple sphincter. Within the mammary gland, *Ltbp1L* is induced exclusively in the ductal luminal epithelium but is silent in alveoli and therefore provides a rare biomarker distinguishing ductal from alveolar luminal lineages. Ltbp1 protein is deposited around basal cells of all ducts and side branches, and lies in close proximity to elastic fibers that exclusively encase the permanent ductal system. *Ltbp1* is prominently upregulated during involution, with kinetics similar to that reported for TGFβ3, suggesting important functions in gland remodeling.

Methods
Mice
Ltbp1L^{lz/+} mice, were generated by Regeneron Pharmaceuticals, Inc., Tarrytown, NY. VelociGene methods [41] were used to recombineer a bacterial artificial chromosome (BAC) clone, such that a region extending from the 165th codon of murine *Ltbp1L* in exon 2 through the remainder of exon 2 and 7.8 kb into the downstream intron, was replaced by homologous recombination with an expression cassette comprising the transmembrane domain of ROR1 fused in-frame with the upstream coding sequence of *Ltbp1L*, followed by a stop-transfer sequence, a modified β-galactosidase coding sequence (*lacZ*), a polyadenylation signal and an antibiotic selection cassette flanked by *loxP* sites [42] (see Figure 1). The modified BAC, was linearized, producing 5′ and 3′ homology arms of approximately 150 kb and 30 kb flanking the deletion, and electroporated into SvEv129/C57Bl6/F1-derived hybrid embryonic stem (ES) cells. Targeting of ES cells and the germline transmission were confirmed by a quantitative reverse transcriptase PCR (qRT-PCR) assay that scored for the loss of one of the native *Ltbp1L* alleles [41]. The *neo*^R cassette was removed by crossing with mice expressing Cre recombinase in the germ-cell lineage and the knockout was confirmed by northern and western analysis [20]. Ltbp1L^{lz/+} mice on a mixed C57Bl6/129 background were rederived into the Skirball animal facility and crossed onto an FVBN strain background by breeding through nine generations. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of New York University School of Medicine.

Carmine staining of mammary whole mounts revealed no differences between *Ltbp1L*^{lz/+} mice and wild-type littermates in ductal elongation, branching, alveolar development or involution. Pups from both genotypes faired
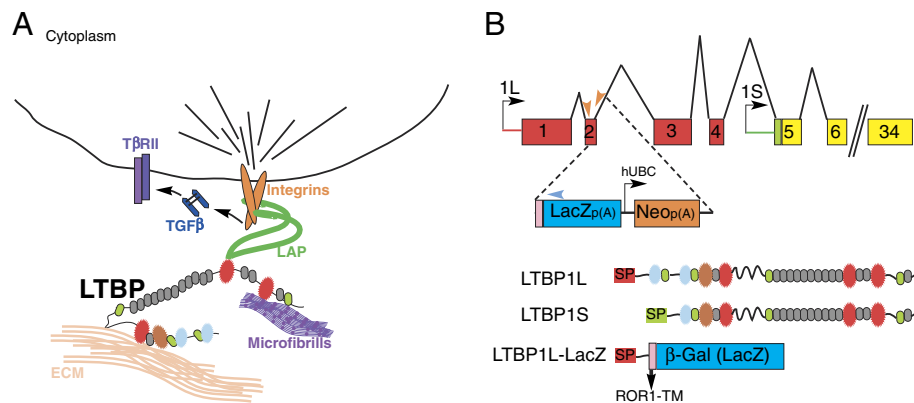
Figure 1 Latent TGFβ binding protein (Ltbp) function and reporter construct. (A) Ltbp1 sequesters TGFβ ligand encased by its latency associated propeptide (LAP) within the extracellular matrix (ECM). Integrins also interact with LAP. Cytoskeletal tension on integrins stretches LAP and releases TGFβ locally to activate the TGFβ receptor II (TβRII). **(B)** Two Ltbp1 isoforms (1L and 1S) are transcribed from distinct promoters (black arrows, top). The first four exons (red boxes) encode sequence unique to Ltbp1L. The promoter, transcription start site and unique signal peptide sequence for Ltbp1S (green line and box) lie within the 4th intron of *Ltbp1L*. Black lines indicate introns. The targeted deletion of *Ltbp1L* replaces codon 165 in exon 2 through 7.8 kb of intron 2 with a cassette comprising the ROR1 transmembrane domain (pink box), a modified *lacZ* gene and polyadenylation signal (blue box) as well as a floxed neomycin gene (orange box) driven by the human ubiquitin binding complex promoter (hUBC) that was removed by cre recombination in embryonic stem cells. Orange and blue arrowheads indicate primers used for genotyping. 1L and 1S isoforms contain a signal peptide (SP), 4-Cys (blue), 8-Cys (red) and epidermal growth factor (EGF)-like repeats (gray and green), and unique 8-cys/EGF hybrid domains (brown). The Ltbp1L-LacZ reporter comprises the signal peptide and the first 165 amino acids of Ltbp1L protein followed by the ROR1 transmembrane domain fused in-frame with β-galactosidase and lacks all functional LTBP1 domains.

equally well in terms of weight gain (data not shown). We concluded that $Ltbp1L^{lz/+}$ mice show no evidence of haploinsufficiency and justified their use to study the regulated expression of $Ltbp1L$ during mammary development. Staging of pregnancy and embryos were performed by daily checking of vaginal plugs, with noon of the day of the plug considered day 0.5. Embryonic stages were confirmed by determining the degree of limb development as indicated in Theiler's classification of mouse development (*The Atlas of Mouse Development*, MH Kaufman).

Genotyping

Mice and embryos were screened by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining of tails and confirmed by PCR analysis. Genomic DNA was prepared from 0.5 cm of tail by digesting overnight in 0.5 ml digestion buffer (50 mM Tris–HCl pH 7.4, 100 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 0.5% SDS, 200 μg/ml proteinase K). Then 150 μl of 5 M NaCl was added and the digest was agitated for 15 minutes on a rotator: 500 μl of supernatant was collected after centrifugation at 14,000 G for 15 minutes, and subjected to two rounds of ethanol precipitation. The final pellet was resuspended in 200 μl TE (10 mM Tris–HCl pH 7.4, 1 mM EDTA) and 1 μl was added to a 20-μl PCR. Thirty cycles of PCR (94°C, 58°C and 72°C for 1 minute each) were carried out. The wild-type *Ltbp1L* allele was detected by amplification of a 430-bp band using forward 5′-CTTAGTTCCTCCATCCTTCC-3′ and reverse 5′-CAGA

CTTCACCTTCCCAGGG-3′ primers. The Ltbp1L$^{lz/+}$ knock-in allele was detected in a separate reaction using the forward primer listed above and a reverse primer 5′-GTCTGTCCTAGCTTCCTCACTG-3′ (see Figure 1B arrowheads) to amplify a 440-bp product. The gender of embryos was determined by amplification of the *Sry* gene on the Y chromosome (forward primer: 5′-GAGAGCATGGAGGGCCAT-3′ and reverse primer: 5′-CCACTCCTCTGTGACACT-3′). Amplification products were resolved by electrophoresis on 2% agarose gels run for 30 minutes in TAE electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA).

X-Gal staining of embryos and mammary gland whole mounts

Embryonic day (E) 10.0 to E15.5 embryos were dissected and fixed in 4% paraformaldehyde (PFA) (Sigma Aldrich, St Louis, MO, USA) prepared in PBS for 20 to 50 minutes depending on the stage. Skin with attached mammary fat pads was removed from E16.5 to E18.5 embryos and stretched carefully on cardboard, and mammary glands from adult mice were dissected and flattened onto glass slides then fixed in 4% PFA for 30 minutes. Following fixation, samples were washed 4 × 15 minutes with rinse buffer (2 mM MgCl$_2$, 0.1% sodium deoxycholate, 0.2% NP40 prepared in PBS) and stained in X-Gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal, Denville Scientific, South Plainfield, NJ, USA) prepared in rinse

buffer) at room temperature for 2 to 3 h. After staining, samples were rinsed twice in PBS and post-fixed in 4% PFA overnight at 4°C, dehydrated through an ethanol gradient (2 × 10 minutes in 70%, 95%, and 100% ethanol), then placed in Carnoys's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) followed by Citrisolv reagent (Fisher Scientific, Pittsburgh, PA, USA) to clear the fat.

Whole-mount carmine staining
X-Gal stained mammary glands were rehydrated in a reverse-graded series of ethanol washed in water and then stained for 1 h in carmine solution diluted 1:5 in water. Carmine was prepared by boiling 1 g carmine alum and 2.5 g aluminium potassium sulphate in 500 ml of water for 20 minutes followed by filtration. The glands were dehydrated in a graded ethanol series, cleared in Carnoy's solution, placed in Citrisolv for 30 minutes, and mounted in Cytoseal (VWR, Radnor, PA, USA). Glands were then viewed using a Zeiss Axiovert (Oberkochen, FRG) bright-field microscope.

Histology and immunodetection
E10.5-stage embryos were embedded in 10% gelatin, sectioned at 70 μm with a vibratome, and mounted with Fluoromount G (Southern Biotech, Birmingham, AL, USA). Older embryos and mammary glands were processed for X-Gal staining and fixation as described above. Isopropanol was substituted for xylene to prevent diffusion of the X-Gal stain during processing and tissues were embedded in paraffin and sectioned. Sections (4 μm) were placed on Superfrost Plus slides, baked 1 h at 60°C and deparaffinized for 5 minutes in Citrisolv for X-Gal-stained tissues. Tissues were then rehydrated through a reverse gradient of ethanol solutions. For histology, sections were stained with 0.1% solution of Nuclear Fast Red (NFR) (Polyscientific, Bayshore, NY, USA) for 1 minute. Tissues were then dehydrated and dipped in xylene (or Citrisolv in the case of X-Gal-stained tissues) before being mounted in Cytoseal (VWR). For immunohistochemistry (IHC), citric acid antigen retrieval was performed by submerging the slide containing deparaffinized 4-μm sections in 10 mM sodium citrate solution (pH 6.0) and boiling in a microwave at 90% power for 30 minutes, followed by quenching of endogenous peroxidase using 3% hydrogen peroxide. Primary mouse antibodies against smooth-muscle actin (SMA) 1 (1:500, DAKO, Carpinteria, CA, USA), estrogen receptor (1:500, DAKO), p63 (1:1,000 LabVision, Kalamazoo, MI, USA), and rabbit antibodies against Cytokeratin 14 (1:8,000, Covance, Princeton, NJ, USA), Lef-1 (1:100 Cell Signaling, Danvers, MA, USA), androgen receptor (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and guinea pig antibodies against Vimentin (1:1,000, Progen) were added overnight at 4°C.

For IHC, biotin-labeled secondary antibodies (1:1,000) and streptavidin-horseradish peroxidase (HRP) (1:200, Vector Laboratories, Burlingame, CA, USA) were added for 30 minutes each, and colorimetrically detected with diaminobenzidine (Vector Labs). Frozen 5-μm sections were stained with rabbit antibodies against LTBP (Ab39 [43], 1:200, a gift from Dr Carl-Henrik Heldin, Uppsala University, Sweden, and rL1C [44], 1:100, a gift from Dr Lynn Sakai, Portland Shriners Research Center, Portland, OR, USA), tropoelastin (1:500, Elastin Products Company, Inc., Owensville, MO, USA), and mouse anti-SMA, described above, were detected by Cy3-labeled donkey anti-rabbit (Fisher Scientific) and Alexafluor-488-labeled donkey anti-mouse secondary antibodies (Life Technologies Inc, Carlsbad, CA, USA). Bioreagent (4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma Aldrich) was used for immunofluorescent localization of nuclei in confocal images. Elastic fibers were also detected by staining with Wiegert's resorcin-fuchsin for 1 minute [45].

Mammary epithelial cell preparation and flow cytometry
The third, fourth and fifth mammary glands from 8- to 16-week-old virgins were dissected, inguinal lymph nodes were discarded, and the mammary glands were minced between two scalpels into a fine paste. The tissue was dissociated for 6 h at 37°C in collagenase/hyaluronidase solution (catalog number 07912, Stem Cell Technologies Inc., Vancouver, BC, Canada), and further dissociated with 0.25% Trypsin-EDTA and 10 mg/ml dispase (catalog number 07913, Stem Cell Technologies) with 1 mg/ml DNase, before filtering through a 40-μm mesh. Endothelial and hematopoietic lineages were depleted using antibodies to TER119, CD45, CD140a, and CD31 (1:100, Becton Dickenson (BD), Franklin Lakes, NJ), with three separations on an EasySep magnet. Primary antibodies CD49f-PerCP-Cy5.5 (1:200, BD), CD24-PE (1:400, BD), CD29-Pacific Blue (1:200, Biolegend, San Diego, CA, USA), CD61-APC (1:200, CalTag MedSystems, Buckingham, UK), stem cell antigen 1 (Sca1)-phycoerythrin (PE) (1:400, BD) were added for 30 minutes at 4°C. Fluorescein Di-β-D-Galactopyranoside (FDG-gal) loading was performed after primary antibody staining, according to the manufacturer's instructions (FluoReporter Kit, Life Technologies, Green Island, NY, USA). Flow cytometry was performed on a BD LSRII or BD FacsCalibur, and analyzed using FlowJo v8.7.

RNA isolation and qRT-PCR analysis
The fourth and fifth pair of mammary glands were harvested from wild-type mice at different stages of postnatal mammary development, dissected and snap-frozen in liquid nitrogen. A block of tissue approximately 0.5 × 0.5 × 0.5 cm was homogenized for 5 minutes in 1 ml of TRI-Reagent (Life Technologies) using a hand-held tissue

homogenizer (Kinematica, Lucerne, Switzerland), then mixed with 200 µl of chloroform and centrifuged at 14,000 G for 15 minutes to eliminate protein debris. The upper aqueous phase was mixed with an equal volume of 70% ethanol and passed through a Qiagen RNeasy mini spin column by a brief 15 sec centrifugation at 8,000 G at room temperature. Total RNA bound to the column filters was washed in 350 µl of ethanol-containing buffer (RW1 buffer; Qiagen, Valencia, CA, USA) to remove contaminants and incubated in 10 µl of RNase-free DNase I enzyme (273 Kunitz units; Qiagen) for 15 minutes at room temperature to ensure digestion of any residual genomic DNA fragments. The columns were washed according to the manufacturer's instructions in ethanol-containing buffers (RW1 and RPE buffers; Qiagen). Total RNA was eluted in 50 µl of RNase-free water, and its concentration was analyzed by Nanodrop measurement. Reverse transcription was performed using 2 µl of RNA (10 ng/µl) from tissue using the QuantiTect Probe RT-PCR Kit (Qiagen; catalog number 204443). Real-time analysis was performed using the Taqman Gene Expression Assay (Applied Biosystems by Life Technologies; catalog number 4331182) for mouse *Ltbp1* (Mm00498255_m1), *Ltbp1L* (Mm01226402_m1 spanning exons 1 and 2), and *Ltbp1S* (custom assay with forward primer: 5′-TTCCAAGGCAA GTTCATGGATA-3′, within intron 4; reverse primer: 5′-AGGAGTAGAGGCAGACAGAGAAAGA-3′, within the fifth exon of *Ltbp1* genomic sequence and MGB probe: 5′-6FAM-TAAGCTGATGTGTTTGTTG-3′-MGBNFQ) and mouse β2-microglobulin (Mm00437762_m1). Real-time analysis was performed in the Applied Biosystems ViiA™ 7. Total Ltbp1, Ltbp1L and 1S mRNA levels were normalized to those of mouse β2-microglobulin and plotted as levels relative to tissue from 12-week-old virgins.

Results

Ltbp1L-LacZ expression underlies a route for axillary cell migration and is an early marker of the mammary mesenchyme

We utilized an Ltbp1L$^{lz/+}$ reporter mouse (Figure 1) in an attempt to understand potential physiological roles of LTBP1L [20]. Mammary development begins in mice at E10.5 with the formation of ectodermal ridges between the limbs, termed mammary lines that fragment to form placodes 3 and 4 [46]. Although Ltbp1L-LacZ expression was found between the limbs at this stage in *Ltbp1L$^{lz/+}$* embryos (Figure 2A black arrow), in sections it localized principally to internal viscera (Figure 2B black arrow). Robust Ltbp1L-LacZ expression first appeared at E11.5 to E12.0 in a subaxillary mesenchymal streak (Figure 2C and D, white arrow) abutting mammary placodes 1 and 2 (Figure 2C, red arrows). Intriguingly, ectodermal cells have been shown to migrate along a similar path to form pectoral and thoracic placodes 1 to 3 [47]. Later Ltbp1L-
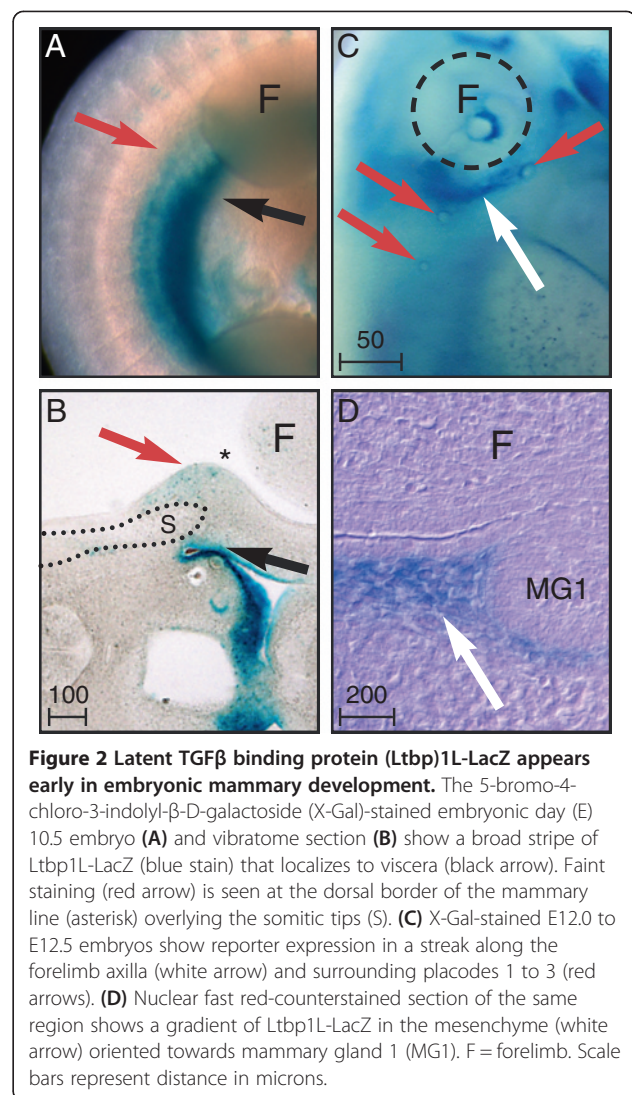

Figure 2 Latent TGFβ binding protein (Ltbp)1L-LacZ appears early in embryonic mammary development. The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained embryonic day (E) 10.5 embryo **(A)** and vibratome section **(B)** show a broad stripe of Ltbp1L-LacZ (blue stain) that localizes to viscera (black arrow). Faint staining (red arrow) is seen at the dorsal border of the mammary line (asterisk) overlying the somitic tips (S). **(C)** X-Gal-stained E12.0 to E12.5 embryos show reporter expression in a streak along the forelimb axilla (white arrow) and surrounding placodes 1 to 3 (red arrows). **(D)** Nuclear fast red-counterstained section of the same region shows a gradient of Ltbp1L-LacZ in the mesenchyme (white arrow) oriented towards mammary gland 1 (MG1). F = forelimb. Scale bars represent distance in microns.

LacZ became intensely expressed around all five buds (Figure 3A-C) and colocalized with well-characterized mammary mesenchyme markers, such as androgen receptor (AR), estrogen receptor (ER), tenascin C and lymphoid enhancer-binding factor 1 (Lef1) (Figure 3D-G) [46]. Thus, during early embryonic mammary development *Ltbp1L* expression underlies a migratory route for epithelial cells and is one of the earliest markers of the inductive mammary mesenchyme.

Mesenchymal *Ltbp1L* activity accompanies nipple induction and persists in smooth-muscle cells and myofibroblasts in the adult

In males, stimulation of androgen receptors at E14.5 induces mammary mesenchymal constriction and atrophy leading to bud loss and failure of nipple formation [48]. In contrast, in females, mammary mesenchyme signaling
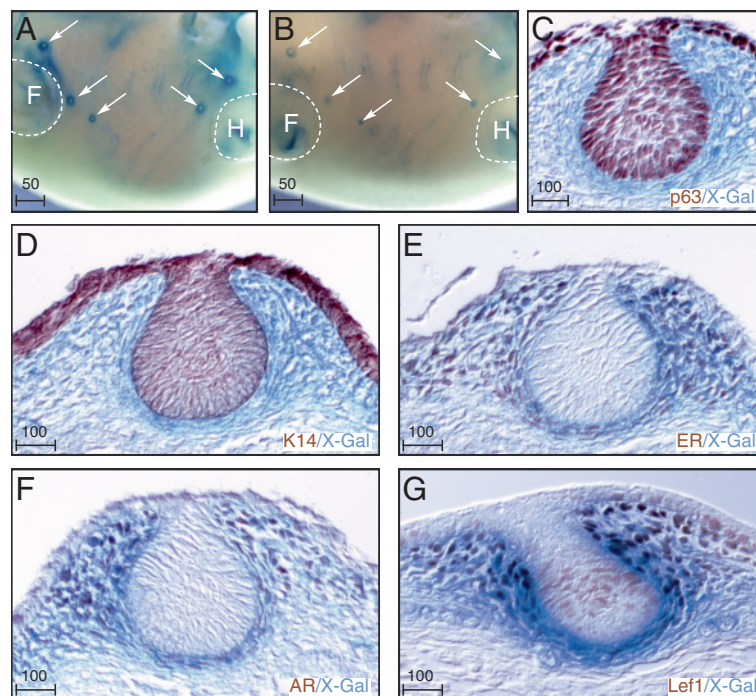
Figure 3 Latent TGFβ binding protein (Ltbp)1L-LacZ is an early marker of mammary mesenchyme. (A) The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained whole mount of embryonic day 14.5 female embryos. Ltbp1L-LacZ is expressed in a halo surrounding all mammary buds (white arrows). Expression is present but at lower levels in male embryos of the same stage (**B**, white arrows), coincident with the onset of sexual dimorphism. Localization of Ltbp1L-LacZ expression (blue stain) with immunohistochemical detection (brown stain) of epithelial markers p63 **(C)** and K14 **(D)**, as well as mammary mesenchymal markers estrogen receptor **(E)**, androgen receptor **(F)**, and lymphoid enhancer-binding factor 1 (Lef1) **(G)**. H = hindlimb, F = forelimb. Scale bars represent distance in microns.

induces ductal morphogenesis, differentiation of nipple epithelium and suppression of hair follicles within the areola [49]. Reflecting this sexual dimorphism, Ltbp1L-LacZ expression was diminished in E14.5 males (Figure 3B) and lost by E15.5 but was maintained in females (Figure 3A), and robustly expressed during nipple induction at E16.5 (Figure 4A, B). Once the nipple shield had formed, reporter expression became restricted to muscle cells of the areola (Figure 4C, D).

Nipples undergo significant postnatal connective tissue remodeling. In virgin and early pregnant mice, the nipple, delimited by germinative epidermal ingrowths, encloses predominantly collagenous connective tissue. During late pregnancy, nipple stromal cells proliferate and synthesize elastin, leading to elastic fiber hypertrophy [50]. Ltbp1L-LacZ was strongly expressed in smooth muscle of the nipple sphincter, located at the base of the areola (Figure 5A-C), which were surrounded by elastic fibers (Figure 5D). *Ltbp1L* was silent within the nipple stroma at most developmental stages (Figure 5A, G, H). However, robust Ltbp1L reporter expression appeared briefly during mid-pregnancy P13.5 within vimentin-positive stromal cells (Figure 5E, F) at the base of the lactiferous duct and directly adjacent to the clefting germinative epithelium. Thus the temporal-spatial expression of *Ltbp1L* appears coincident with the formation of the nipple sphincter and during elastin synthesis by stromal myofibroblasts.

Ltbp1L promoter activity coincides with ductal lumena formation within the embryonic mammary tree

At E16 mammary mesenchymal signaling induces proliferation of a solid cord of cells to form the mammary sprout [46,49]. *Ltbp1L* remained silent within the epithelium at this stage (Figure 4B) but became robustly expressed at around E17.5, in luminal cells coincident with the appearance of microlumen (Figure 6B, D). Intriguingly, reporter expression was absent from the multilayered ductal tips (arrowheads, Figure 6E) and from portions of the lactiferous duct within the nipple that comprise stratified epithelium (Figure 6D). Thus, *Ltbp1L* is induced only when the lactiferous duct enters the fat pad and differentiates into a bi-layered tube comprising a simple epithelial luminal lining surrounded by molecularly distinct basal cells.

Ltbp1 mRNA is dynamically modulated during postnatal development

Mammary development continues postnatally during puberty and is completed only after a first pregnancy. To determine if *Ltbp1* was expressed during the postnatal period we isolated total RNA from mammary glands
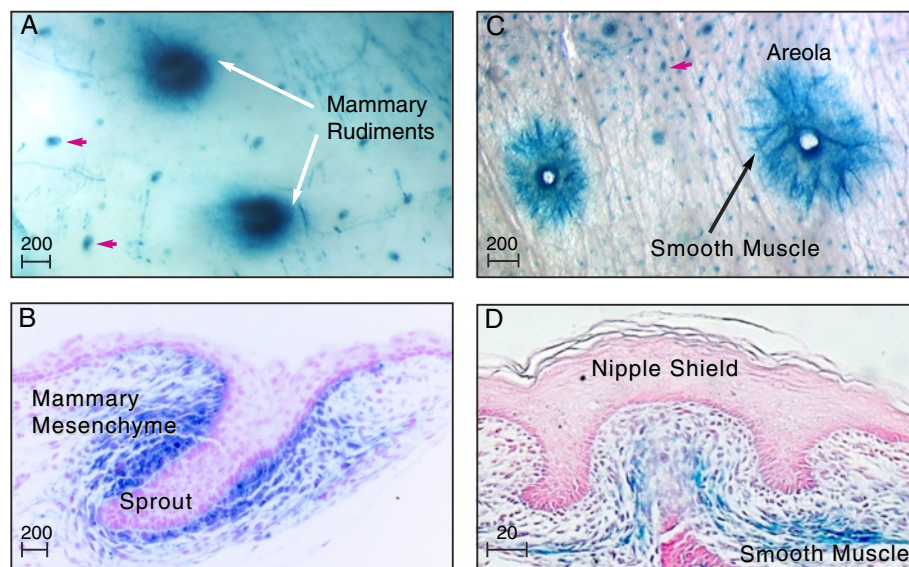
Figure 4 Mesenchymal *Ltbp1L* activity is upregulated coincident with embryonic nipple formation. (A) The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained skin whole mount (embryonic day16.5) shows robust Ltbp1L-LacZ expression (blue stain) in the mammary rudiments (white arrows) and developing hair follicles (red arrowheads). **(B)** Nuclear fast red (NFR)-counterstained section of the E16.5 mammary sprout shows strong focal Ltbp1L-LacZ expression in mammary mesenchyme at the onset of nipple induction. **(C, D)** Postnatal day 1 X-Gal-stained skin whole mount **(C)** and NFR-stained section **(D)** show Ltbp1L-LacZ expression in smooth-muscle cells at the base of the nipple shield extending radially into the developing areola. Scale bars represent microns.

of virgin, pregnant and lactating mice as well as from those undergoing post-parous remodeling (involution), and performed qRT-PCR. Total Ltbp1 mRNA was expressed at modest levels in virgin mice, decreased during pregnancy, lost during lactation and robustly upregulated during early involution, peaking at day 3 and returning to that found in virgins after 5 to 7 days (Figure 7A). Ltbp1S and 1 L showed a similar trend, however Ltbp1L rose in a more pronounced fashion at day 3 (Figure 7B).

Ltbp1L is induced in ductal luminal cells and distinguishes them from alveolar lineages

To determine more precisely where the *Ltbp1L* promoter is activated during postnatal mammary development, we examined Ltbp1L-LacZ expression in whole mounts and histological sections. In pubertal mice a balance of proliferation and apoptosis within outer cap and inner multilayered body cells of the bulbous terminal end buds (TEBs) generates the permanent ductal tree and creates a lumen in the subtending ductal system. X-Gal-stained whole mounts revealed Ltbp1L-LacZ expression lining the lumen of the TEB (Figure 7C). Reporter expression was notably absent from the vast majority of body cells, which are considered to be actively proliferating luminal precursors (Figure 7D).

Ltbp1L-LacZ was expressed prominently in luminal cells of the permanent ductal system (Figure 8A, B). To further characterize *Ltbp1L* activity within the luminal

lineage we utilized a fluorescent β-Galactosidase substrate, FDG-Gal to detect Ltbp1L-positive cells by flow cytometry. Mammary stromal, basal, and luminal subpopulations can be separated by their differential expression of CD24, CD49f and CD29 (Figure 8C top panel) [51]: 65% of $CD24^{high}CD49f^{low}$ and $CD29^{low}$ luminal cells (Figure 8C middle panel and 8D respectively) were FDG^+ and therefore expressed Ltbp1L-LacZ. Interestingly, 35% of the luminal cell population was negative (Figure 8C bottom panel), consistent with our observation of a punctate X-Gal staining pattern in some whole mounts (Figure 8A). Luminal cells can be further defined into mature and progenitor populations by their expression of CD61, a marker of integrin β3 that is highly expressed in luminal progenitors and Sca1 [51]. The majority of FDG^+ cells were $Sca1^+$ (Figure 8E) and $CD61^-$ (Figure 8F), but a small percentage was $CD61^+$. Collectively these data indicate that *Ltbp1L* is induced in a subset of luminal progenitors and mature luminal cells of the permanent ductal system.

Pregnancy initially induces extensive arborization of the mammary ductal tree. Alveolar clusters form on the tip of each side-branch during mid-pregnancy and undergo secretory differentiation during late pregnancy in preparation for lactation. Mammary whole mounts taken during early, mid and late pregnancy showed Ltbp1L-LacZ expression throughout the permanent ductal system and within the newly developing transient side branches (Figure 9A-D). In striking contrast, *Ltbp1L*
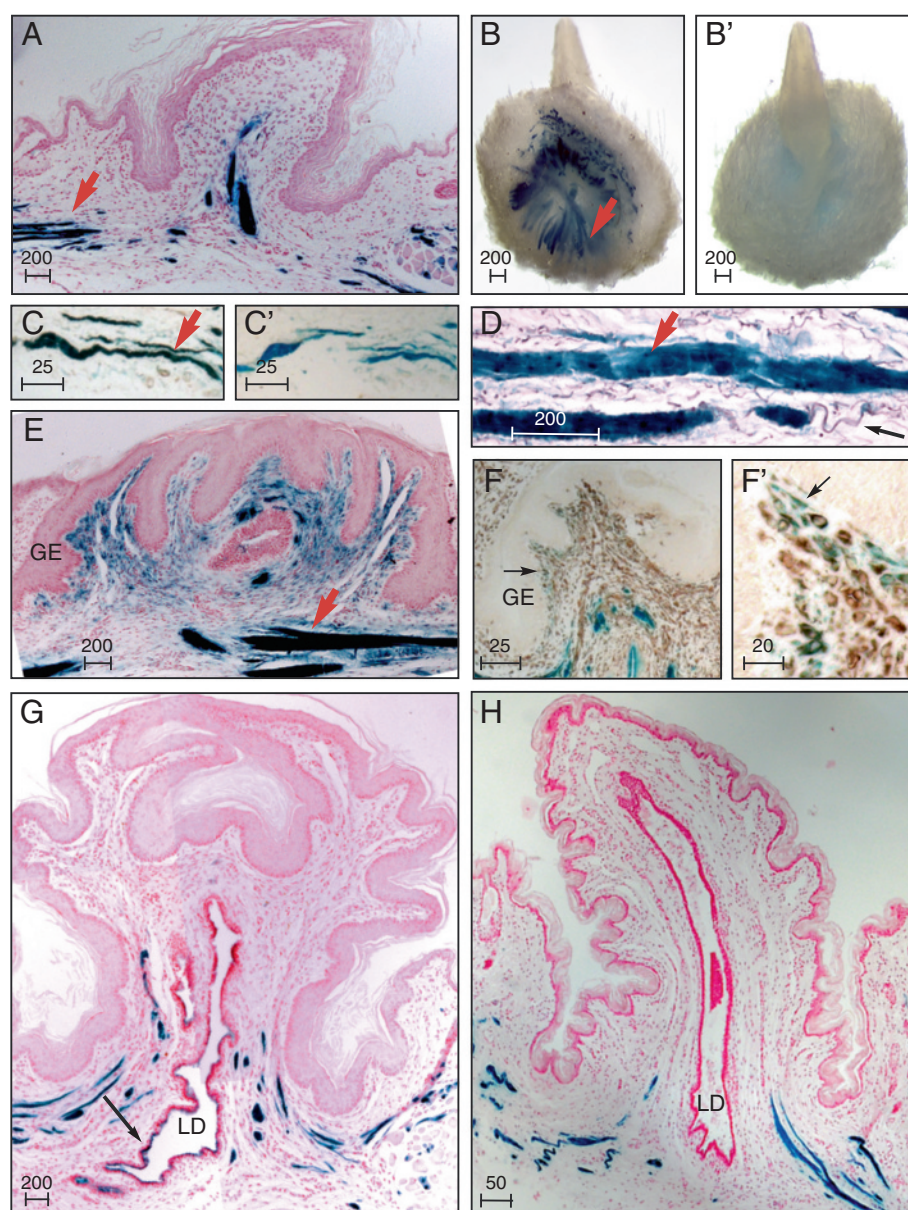
Figure 5 Sustained latent TGFβ binding protein (Ltbp)1L-LacZ expression in the specialized stroma of the adult nipple. (A-E) Robust Ltbp1L-LacZ expression was detected throughout postnatal development by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining (blue stain) in bands of smooth-muscle cells (red arrows) at the base of the nipple underlying the areola in the region of the nipple sphincter **(A)** Histological section through a nipple of a virgin mouse, counterstained with nuclear fast red (NFR). **(B, B')** Nipples from a pregnant (P14.5) mouse are shown from below **(B)** and above **(B')**. **(C)** Immunohistochemical detection of SMA 1 (brown stain) colocalizes with Ltbp1L-LacZ in areola smooth muscle, P13.5. Secondary antibody control **(C')**. **(D)** Resorcin-fuchsin stain detects elastin fibrils (black arrow to deep purple stain) encompassing Ltbp1L-LacZ-expressing smooth muscle. **(E)** NFR-stained section shows Ltbp1L-LacZ upregulation in nipple stromal cells during pregnancy, P13.5. **(F, F')** Colocalization of Ltbp1L-LacZ expression (black arrow to blue stain) with immunohistochemical detection of vimentin (brown stain) in stromal cells underlying the nipple germinative epithelium (GE) just prior to clefting, during pregnancy (P15.5). NFR-counterstained nipple sections from mice during **(G)** pregnancy P16.5 and **(H)** involution. Note the absence of X-Gal staining in the region of the lactiferous duct (LD) within the nipple in **G** and **H** and acquisition of reporter expression as the LD enters the fat pad at the base of the nipple (see black arrow in **G**). Scale bars represent distance in microns.

remained silent within developing and differentiating alveoli throughout pregnancy (Figure 9C, D). Histological sections through p16.5 alveolar clusters confirmed that Ltbp1L-LacZ expression was restricted to ducts and side branches (Figure 9E, F) and absent from alveolar milk-producing cells that contained large lipid droplets (Figure 9F). Thus *Ltbp1L* is a rare and highly specific marker distinguishing ductal from alveolar luminal lineages.
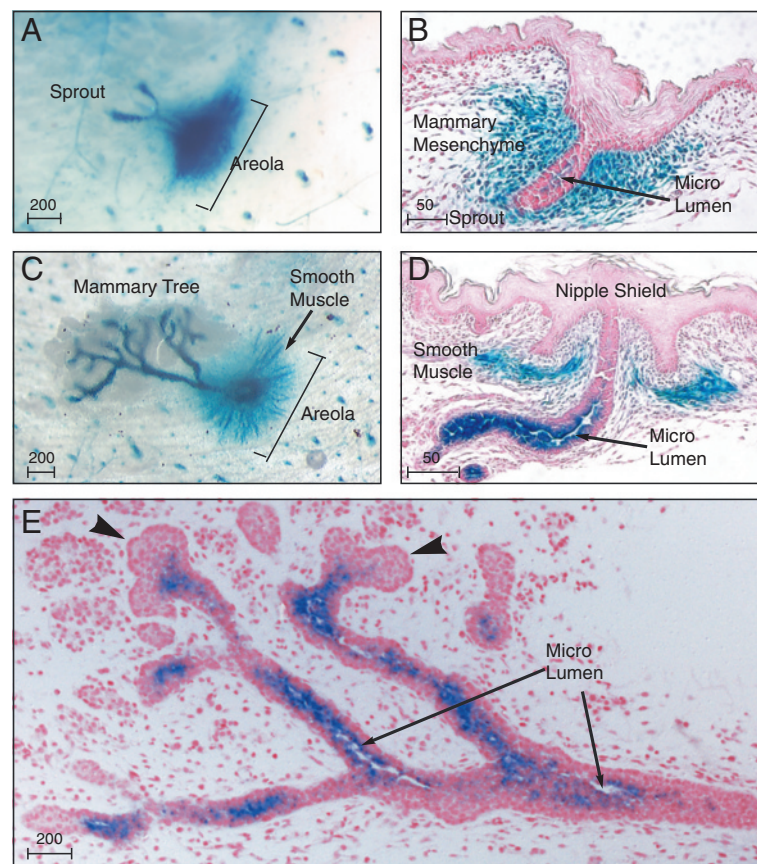
Figure 6 Latent TGFβ binding protein (Ltbp)1L-LacZ appears at the onset of lumen formation. (A) The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained whole mounts **(A, C)** and sections counterstained by nuclear fast red **(B, D, E)** at embryonic day (E) 17.5 **(A, B)**, postnatal day 1 P1 **(C, D)** and E18.5 **(E)**. Note Ltbp1L-LacZ appears as microlumen form within the mammary sprout **(B**, **D**, **E** arrows). Ltbp1L-LacZ is absent from the bulbous multilayered tips **(E**, arrowhead). Ltbp1L-LacZ is sustained in the mammary mesenchyme **(A, B)** and developing smooth muscle cells of the nipple areola **(C, D)**. Scale bars represent distance in microns.

Ltbp1L activity is dramatically upregulated during involution

During lactation luminal cells of both ducts and alveoli undergo secretory differentiation. Ltbp1L-LacZ expression was undetectable in whole mounts and sections at this stage (Figure 10A, B) consistent with the relative diminishment of Ltbp1 mRNA expression at this stage in qRT-PCR analysis (Figure 7A, B). Milk stasis and ductal distension trigger an initial phase of involution involving cell death that is reversible if suckling resumes [37,52]. After 48 h, however, involution proceeds irreversibly with collapse and removal of transient alveolar and side-branch structures. Throughout this process the permanent ductal system and resident stem cells are protected from destruction. Within 24 hours of pup weaning Ltbp1L-LacZ expression appeared along the main ducts and distended primary side branches (Figure 10C, D). In sections, the reporter expression appeared in a discontinuous pattern within a subset of luminal cells (Figure 10D) and was absent from alveoli, which remained morphologically distended. By 72 h, as the

alveoli collapsed and were undergoing clearance, Ltbp1L-LacZ became robustly expressed within remaining luminal epithelia (Figure 10E, F). This sharp increase in LTBP1 expression around the transition to irreversible involution was confirmed by qRT-PCR where Ltbp1, 1 L and 1S mRNA peaked at 72 h (Figure 7A, B). Collectively these results show that both forms of *Ltbp1* are transcriptionally regulated throughout mammary development in a highly dynamic temporal and spatial manner and are maximal during involution.

Ltbp1 and elastin encase the mammary ductal system

Having identified the spatial pattern of *Ltbp1L* promoter activity and expression levels of both Ltbp1 mRNAs we next sought to determine the localization of the secreted Ltbp1L protein. We first examined sections of involuting glands by immunofluorescence and found Ltbp1L localized in microfibrillar strands surrounding ducts (Figure 10G). Resorcin-fuchsin detected elastic fibers in a similar periductal organization (Figure 10H). We next examined Ltbp1L
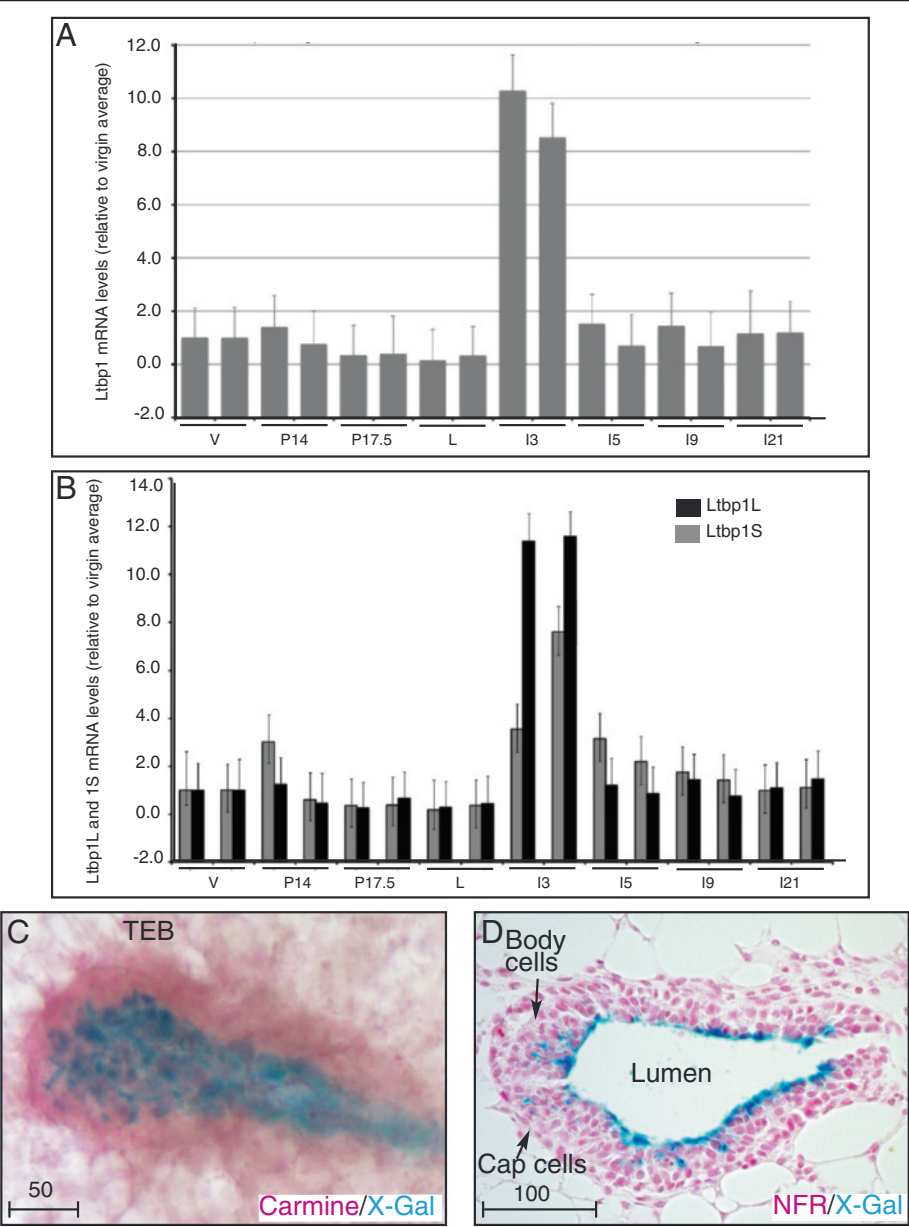
Figure 7 *Latent TGFβ binding protein* (Ltbp)*1L* expression in the postnatal mammary gland. (A) Ltbp1 mRNA expression is highly elevated during early involution peaking 3 days after forced pup weaning. **(B)** Ltbp1L (black bars) and Ltbp1S (gray bars) are most abundant during early involution day 3. Total RNA from mammary gland tissues, harvested from various developmental time points (12-week virgin (V), 14 days pregnant (P14) and 17.5 days pregnant (P17.5), lactating (L), involution days 3 (I3), 5 (I5), 9 (I9) and 21 (I21)), was reverse transcribed and subjected to qRT-PCR. Ltbp1 **(A)** mRNA levels as well as those of Ltbp1L and Ltbp1S isoforms **(B)** were normalized to β2-microglobulin expression and plotted as levels relative to tissue from 12-week-old virgins. Error bars indicate standard deviation of the cycle threshold (Ct) values (n = 4). mRNA levels from two representative mouse samples for each time point are shown on the graphs. **(C, D)** Ltbp1L-LacZ (blue stain) expression in cells bordering the lumen of the terminal end bud (TEB). **(C)** In carmine-5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained whole mounts, Ltbp1L-LacZ localizes to the internal portion of TEB and is surrounded by non-expressing cap and body cell layers. Note the punctate appearance of X-Gal staining demonstrating that Ltbp1L-LacZ is in a subset of luminal cells. **(D)** Sections of the same 5-week-old virgin stained with nuclear fast red shows Ltbp1L-LacZ expression in cells bordering the lumen. Scale bars represent distance in microns.

and elastin organization at earlier stages (Figure 11). In pubertal glands SMA antibodies detected the basal cell layer of ducts (Figure 11A, B) but was absent from the contiguous cap cell layer of TEBs. Ltbp1 antibodies showed extensive regions of colocalization with SMA-positive basal cells along ducts but was also absent from the SMA-negative cap cell
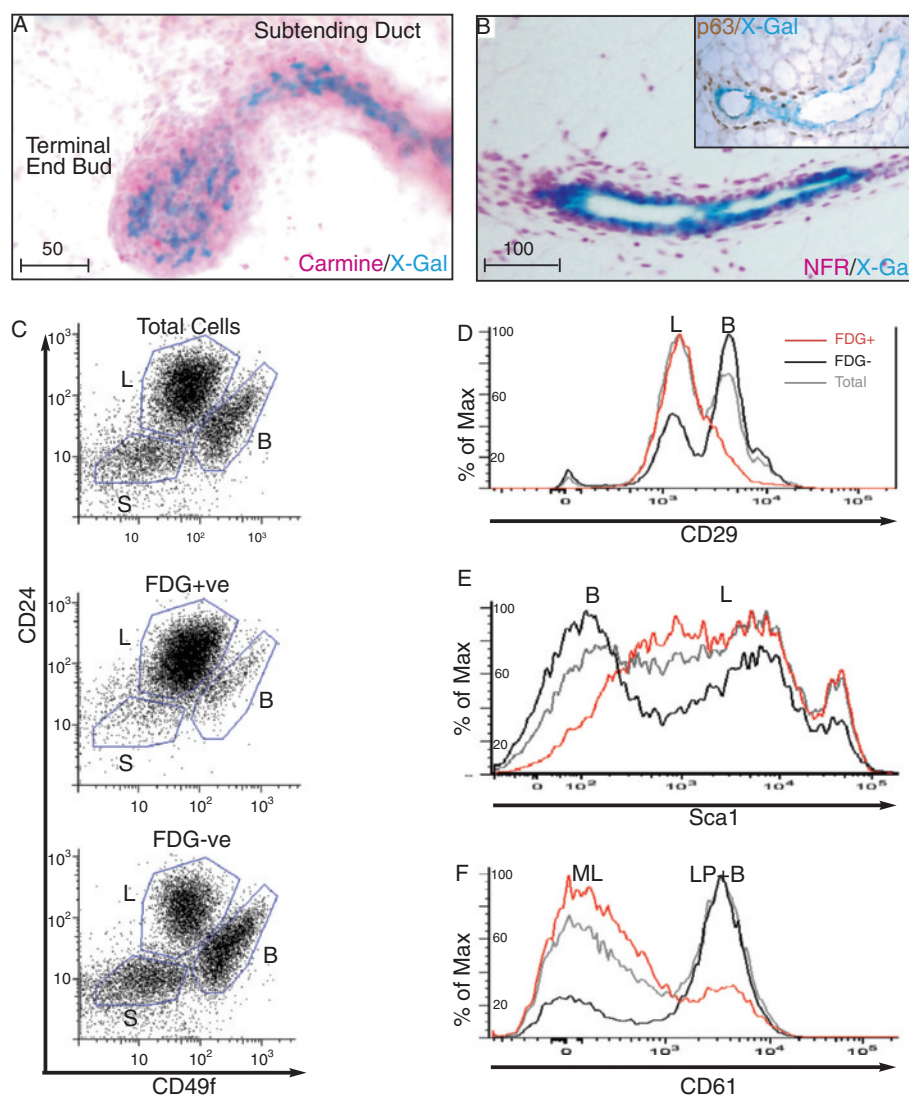
Figure 8 A major subset of ductal luminal cells show *Ltbp1L* activity. (A) Carmine/5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained whole mount of the terminal end bud, **(B)** nuclear fast red-stained section of the mature duct show Ltbp1L-LacZ expression in luminal cells, basal cells are visualized by immunohistochemcal detection of p63 (inset). Flow cytometry gating for FDG-Gal$^+$ (Ltbp1L-LacZ)$^+$ cells enriches for the luminal CD24$^+$CD49^{LO} compartment (**C**, top panel). Ltbp1L-LacZ marks 65% of total luminal cells (**C**, middle panel), and 35% of luminal cells are Ltbp1L-LacZ-negative (**C**, bottom panel). Gates represent stromal *S*, luminal *L*, and basal *B* populations from two mature virgin mice. **(D-F)** Histograms show Ltbp1L-LacZ -positive cells (red) are CD29^{LO} **(D)**, Sca-1^{HI} **(E)** and CD61^{LO} **(F)**. Data represents n = 2 replicates for all experiments. Peaks represent luminal *L*, basal *B*, mature luminal *ML* and luminal progenitors *LP*. Scale bars represent distance in microns.

layer of TEBs indicating that Ltbp1 is deposited in close apposition to differentiated myoepithelial cells (Figure 11A-B). Weak Ltbp1 staining was seen in a few body cells of the TEB. The ductal system was also encased by a thick mesh of elastic fibers detected by anti-tropoelastin (Figure 11C). The elastic fibers, however, localized more distantly from the basal cell layer than Ltbp1. In glands from pregnant mice, Ltbp1 surrounded both the permanent ductal system and temporary side branches but was absent from alveolar clusters (Figure 11D). In contrast elastic fibers were restricted to the permanent ductal system (Figure 11E).

Discussion

The importance of TGFβ signaling for mammary physiology and pathology has been well documented however the factors that regulate TGFβ presentation and activation are less well-understood [53]. Although LTBPs determine the spatial deposition of latent TGFβ and thus define the coordinates for its subsequent activation, surprisingly nothing is known about them in normal mammary gland. Here we show that *Ltbp1* is dynamically and focally regulated throughout mammary development. The major findings of our study are that 1) Within the mammary epithelium,
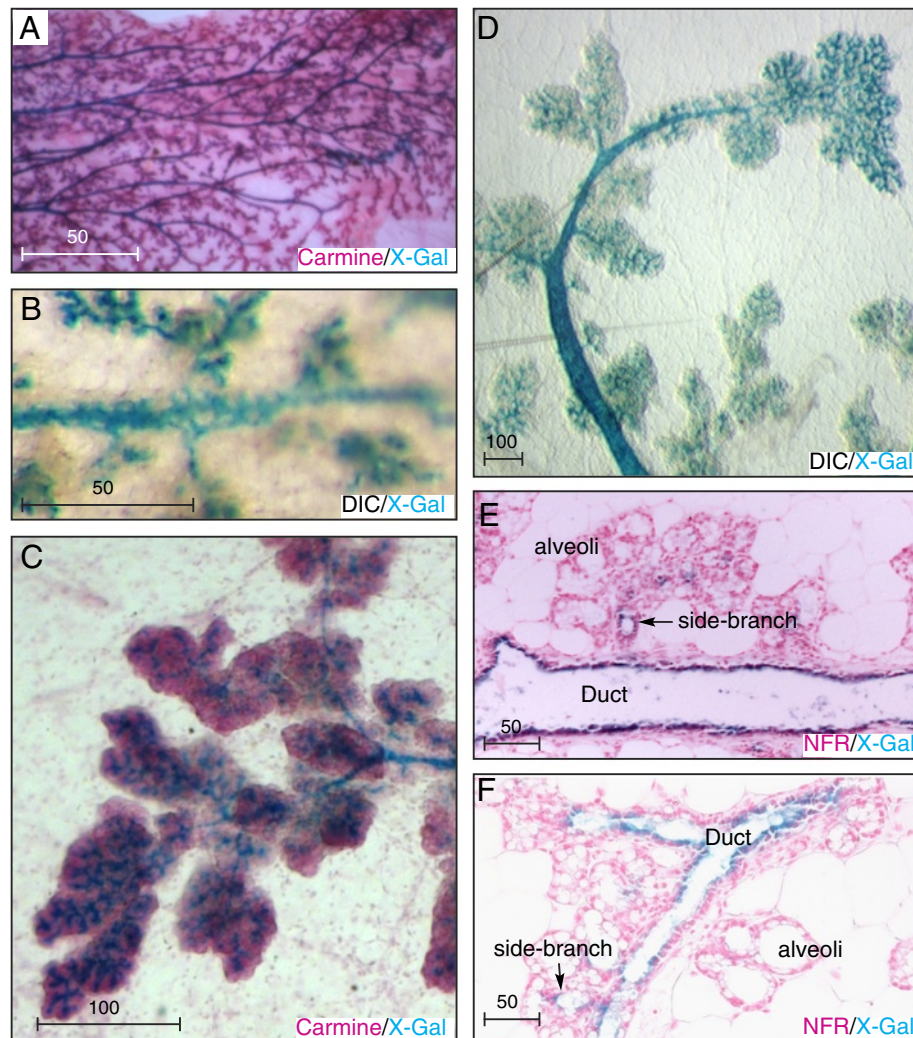
Figure 9 Latent TGFβ binding protein (Ltbp)1L is silent in secretory alveoli during pregnancy. (A-D) The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained whole mounts show Ltbp1L-LacZ (blue stain) is expressed in ducts and side branches at all stages of pregnancy, but absent from alveoli. **(A)** Carmine/X-Gal-stained whole mount P13.5. **(B)** Ltbp1L-LacZ is expressed in a reticular staining pattern demarcating a subset of ductal cells. **(C)** Carmine/X-Gal-stained whole mount P14.5 **(D)** X-Gal-stained whole mount P14.5. **(E, F)** nuclear fast red(NFR)/X-Gal-stained sections P16.5 show Ltbp1L-LacZ is expressed in ducts but not in adjacent alveoli. Scale bars represent distance in microns.

Ltbp1L is transcribed exclusively by ductal luminal cells and distinguishes them from the alveolar luminal lineage; 2) Ltbp1 protein and elastic fibers exclusively encase the ductal system; 3) *Ltbp1L* and *1S* are upregulated during involution, a developmental window linked to high risk for breast cancer promotion; and 4) *Ltbp1L* is induced in mammary mesenchyme and sustained in the smooth-muscle cells of the nipple sphincter.

Ltbp1L is induced in embryonic mammary mesenchyme and persists in nipple sphincter cells

Ltbp1L-LacZ is first expressed in an arc around the fore-limb. This pattern is intriguing in light of reports that mammary precursors destined for placodes 1 to 3 migrate along a similar path [47]. It is well known that

TGFβ signaling promotes EMT and motogenesis. Thus, *Ltbp1L* expression may designate a migratory route and potentially stimulate ectodermal cell migration by presenting a focal source of TGFβ. *Ltbp1L* is next upregulated in the specialized mammary mesenchyme, which plays a pivotal role in inducing mammary morphogenesis and specifying the embryonic nipple and areola [49]. To date there have been no reports of TGFβ involvement in these inductive processes, although other members of the TGFβ family, such as bone morphogenic protein (BMP)4, are known to play critical roles [54,55]. We find that the expression of mammary mesenchymal markers remains unperturbed and embryonic mammary development proceeds normally in *Ltbp1L^{lz/lz}* embryos, indicating that *Ltbp1L* is not essential for mammary
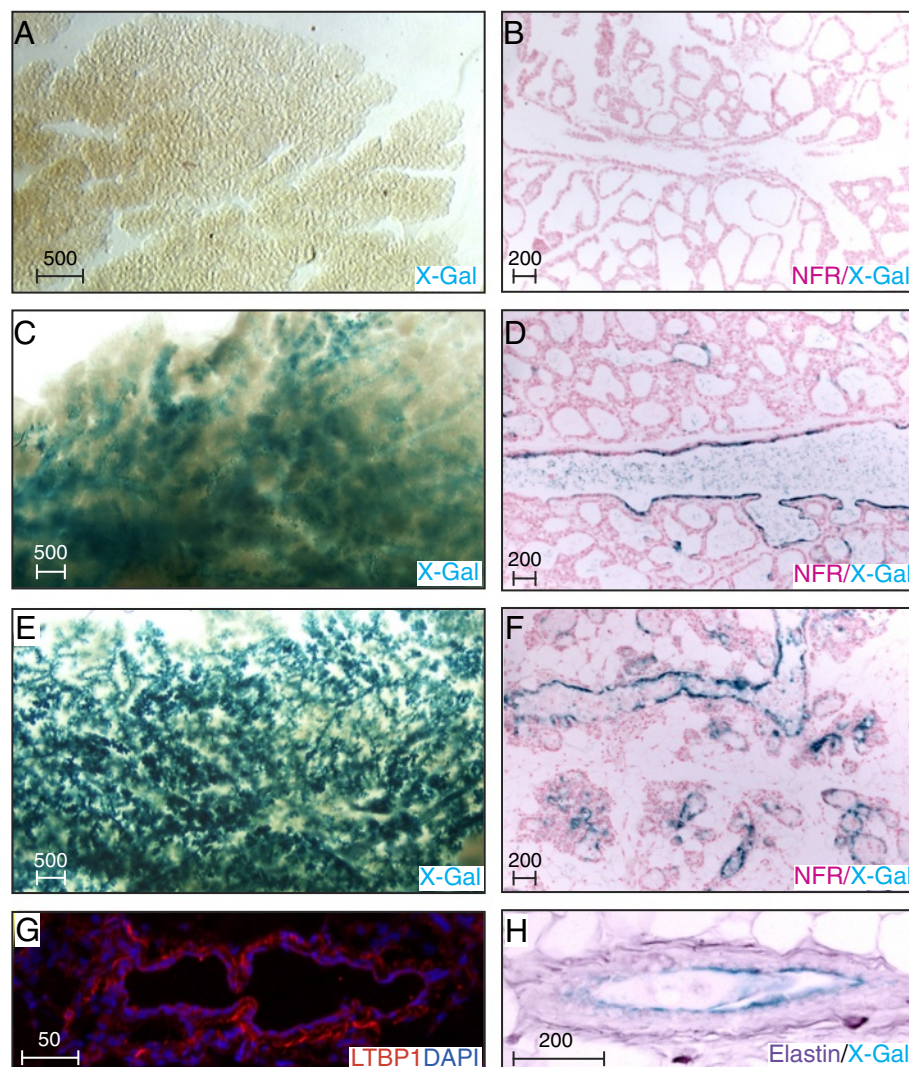
Figure 10 Modulation of latent TGFβ binding protein (Ltbp)1L-LacZ expression during involution. (A, C, E) The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained whole mounts and **(B, D, F)** nuclear fast red (NFR)-stained sections of lactating **(A, B)**, and involuting glands removed 24 h **(C, D)** and 72 h **(E, F)** after forced pup weaning. **(G)** Immunofluorescent detection of frozen mammary glands isolated from mice at involution day 5 using anti-LTBP1 (red) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) to detect nuclei. **(H)** Elastic fibers (purple), identified by resorcin-fuchsin staining, on X-Gal-stained paraffin-embedded sections of mammary gland harvested from mice at involution day 21 shows periductal arrangement of fibrils encasing the permanent duct. Scale bars represent distance in microns.

mesenchyme specification or inductive function. These results do not, however, preclude the possibility that the products of *Ltbp1S*, which is expressed from an independent promoter, or other *Ltbp* genes may compensate [56]. Alternatively Ltbp1L may function at later stages in the differentiation of these cell types. Ltbp1L-LacZ expression persists within smooth muscle cells aligned in radial arrays under the areola, which facilitate nipple projection and regulate the nipple sphincter during milk let-down. There have been no studies on TGFβ in the nipple, however, misexpression of Wnt5a, a target gene of TGFβ, has been shown to impair milk ejection, supporting the concept that specific levels of TGFβ

signaling may be critical for nipple function [57]. We also observe strong Ltbp1L-LacZ expression in myofibroblasts during mid-pregnancy when the stroma synthesizes elastin to provide structural support for the lactiferous duct [58]. Whether Ltbp1L functions to reinforce the surrounding elastic fibers, and/or serves in a mechanosensory capacity between TGFβ signaling and the establishment of the unique nipple stroma, remains to be determined.

Ltbp1 and ductal cell fate

Ltbp1L activity is a consistent marker of the ductal luminal lineage, appearing in the embryo at the first sign
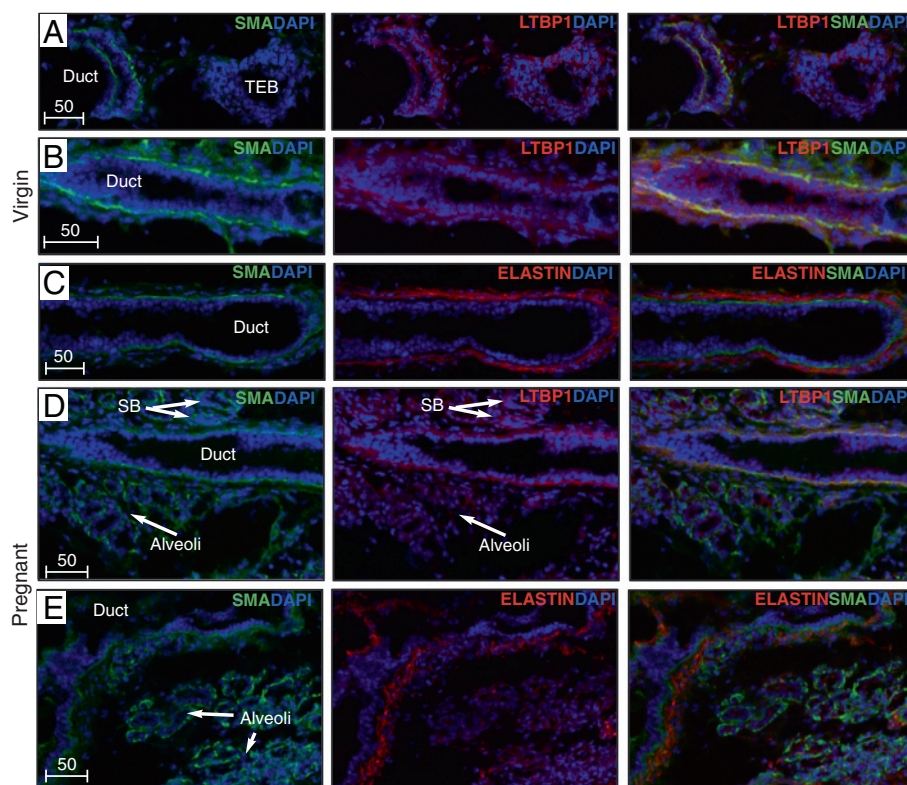
Figure 11 Localization of latent TGFβ binding protein (Ltbp)1 and elastin in the pubertal and pregnant mammary gland.
Immunofluorescent detection of Ltbp1 **(A, B** and **D)**, elastin **(C, E)** and smooth-muscle actin (SMA) **(A-E)** in frozen sections of mammary glands isolated from 6-week-old pubertal **(A-C)** and 14.5-day pregnant mice **(D, E)**. Anti-SMA detected by Alexa-488 secondary antibodies (green) localized basal myoepithelial cells of mature ducts but not cap cells of the terminal end bud (TEB) and robustly stained basal cells of alveoli. Anti-Ltbp1 detected by Cy3-coupled secondary antibodies (red in center panels) in virgin **(A, B)** and pregnant glands **(D)** detected Ltbp1 deposited in close apposition to the SMA-positive basal cell layer (yellow in right merged panel). In contrast the cap cell layer that surrounds the TEB was negative with both anti-SMA and anti-Ltbp1 antibodies. Anti-tropoelastin detected by Cy3 coupled secondary antibodies (red in center and right panels) in virgin **(C)** and pregnant **(E)** glands detects elastic fibers surrounding the basal layer. Both Ltbp1 and elastic fibers are excluded from alveolar clusters. Frozen sections were viewed under a Carl Zeiss Confocal LSM510 microscope at 20× magnification and images were acquired using Zen Software version 2010. Scale bars represent distance in microns. DAPI, 4',6-diamidino-2-phenylindole.

of ductal canalization. This specificity is maintained throughout pubertal development and pregnancy where it serves as a rare marker distinguishing ductal from alveolar luminal cells. Transplantation studies have suggested that ductal and alveolar progenitors are distinct, but little is known about differences between mature ductal and alveolar luminal cell-types [59]. *Ltbp1L* is active in approximately 65% of luminal cells but silent within the inner body cells of the TEB, which are thought to be a proliferative progenitor population. It is upregulated within mature CD61$^-$Sca1$^+$ cells in the subtending duct and within a small subpopulation of CD61$^+$ luminal progenitors, which we speculate may generate side branches during pregnancy. Previous studies have implicated TGFβ signaling in suppressing proliferation of luminal populations and maintaining the potency of basal stem cell populations [34,36,60]. Our results show that Ltbp1 protein is deposited in close apposition to basal cells encasing the ductal system and thereby positioning TGFβ to carry out these functions.

Ltbp1 in the physiology of ductal dilation and distension
The appearance of Ltbp1L-LacZ expression coincident with lumen formation in the embryonic mammary rudiment and in the pubertal TEB suggests Ltbp1 may position TGFβ to generate lumen by inducing apoptosis [61]. TGFβ is a well-known pro-apoptotic cytokine and multiple studies have demonstrated a role for apoptotic factors in lumen formation *in vitro* and *in vivo* [34,37]. However the periductal restriction of Ltbp1 protein in close association with elastic fibers makes this function unlikely and moreover indicates that they participate in some ductal versus alveolar specific process. A distinguishing feature of ducts is that their lumen remain open at all times. Whether Ltbp1 serves to physically support the open ducts by reinforcing their elastic fiber encasement and/or positions TGFβ to monitor ductal

lumenal diameter in a mechanosensory fashion remains to be determined.

Ltbp1L is silenced during lactation and dramatically induced during involution

The most dramatic changes in *Ltbp1* activity occurred with the onset and cessation of lactation. Ltbp1, 1L and 1S mRNA were undetectable during lactation, and Ltbp1L-LacZ expression was lost even from the ducts as the entire epithelium assumed a secretory phenotype and the lumen became engorged with milk. This loss of *Ltbp1L* and 1S expression coincides with a change in the trafficking of latent TGFβ from basolateral secretion as a large latent complex destined for incorporation into the ECM in an Ltbp-dependent manner to apical secretion of small latent complex into milk, which functions to promote IgA production and induce oral tolerance in the newborn [62].

Ltbp1L is dramatically induced during involution. Involution is a biphasic event, marked by distinct biological processes. For up to 48 h after weaning the process is reversible and characterized by alveolar apoptosis. After this point it becomes irreversible, as protease-mediated matrix remodeling leads to alveolar collapse and rebuilding of the ECM, to return the gland to a virgin-like state [63]. Teat-sealing experiments have shown that ductal distension triggers involution even in the presence of circulating lactogenic hormones, highlighting the role of local factors [37]. Our results show that *Ltbp1L and 1S* are induced within 24 h and peak at day 3 of involution, remaining elevated for some days. This pattern is similar to that reported for TGFβ3 in several microarray studies [64,65]. TGFβ3 is upregulated 6-fold within 3 h of weaning and has been implicated as a local factor triggering alveolar apoptosis, however, the mechanism for its activation has not been studied [37]. Whether *Ltbp1* is expressed early enough to facilitate TGFβ3's role in apoptosis remains to be determined. The peak of *Ltbp1* and TGFβ3 induction correlates with the transition to the irreversible stage of involution, suggesting that elevated TGFβ signaling may contribute to this transition. Little is known about the role of TGFβ3 in later involution, though it has been hypothesized to promote fibroblast migration and ECM generation based on the upregulation of wound healing and ECM genes that are targets of TGFβ signaling during this phase [17,63,65]. Alternatively, the localization of Ltbp1 protein along ducts suggests it may function to protect the permanent ductal system and its ductal stem cells from destruction by integrating integrin and TGFβ signaling, which promote cell survival and stem cell potency, respectively [66]. Lastly, our finding that *Ltbp1* expression is dramatically elevated during involution, when taken collectively with the fact that *LTBP1* appears in two metastatic signatures [5,6] and regulates TGFβ, a factor inducing EMT, suggests that LTBP1 may be a prometastatic element in pregnancy-associated breast cancer (PABC). Detected postpartum, PABC is highly aggressive and this feature is thought to result from the action of prometastatic factors in the microenvironment of involuting glands [67]. Thus LTBP1 levels may be worthy of investigation as a risk factor.

Conclusions

In conclusion, our results establish that *Ltbp1* is dynamically regulated during mammary development. The pattern of *Ltbp1L* activity and Ltbp1 protein localization suggest roles in reinforcing elastic support and mechanosensory feedback for mammary ducts and nipple. Currently nothing is known of the role of this important TGFβ regulator in human breast. Its elevation during involution suggests LTBP1 is worthy of further investigation as a prometastatic candidate in PABC.

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